University of Alberta

Synthesis and Immunochemistry of Lewis Y Analogs

by

Lesley Mae Lin Liu ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment

of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

Edmonton, Alberta

Spring 2003

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Abstract

In the last decade, some promising results have emerged from the evaluation of synthetic, anti-cancer, carbohydrate conjugate vaccines. However, there remain large gaps in our understanding of immunology and tumor biology that hamper the rational design of vaccine candidates. In particular, the origin of antibody anti-tumor selectivity has not yet been identified, nor have the structural criteria for optimal immunogenicity in carbohydrate conjugate vaccines been delineated.

Described here is the synthesis of structurally related analogs and conjugate vaccine candidates that incorporate the tumor-associated carbohydrate antigen Lewis Y (Le^y). Also reported are studies using these synthetic compounds to elucidate the relationship between the structure of the immunizing antigen, the epitope recognized by the elicited antibodies, and the selectivity shown by the elicited antibodies for tumor cells over normal cells expressing the same antigen.

Mouse monoclonal antibody B3 is notable for its selective reactivity with Le^y-expressing tumor cells. Frontal affinity chromatography coupled online with mass spectrometry (FAC/MS) was employed to estimate the affinity of B3 for the synthetic Le^y analogs. The results of these experiments show that B3 does bind extended structures with greater affinity than it binds the tetrasaccharide Le^y determinant, thus lending credence to the previously-reported speculation that the binding specificity of anti-tumor monoclonal antibodies (e.g. B3) arises from extended epitope recognition.

The synthesis of the Le^y analogs was extended to the construction of two BSA conjugate vaccine candidates. In contrast to previously-reported, synthetic Le^y-based conjugate vaccines that focus either solely on the minimal carbohydrate determinant or on mimicking mucins, the vaccine candidates described here present the Le^y determinant in the context of a glycosphingolipid. The aim of this study was to determine whether

extending the immunizing structure to more closely mimic the natural glycolipid antigen would elicit polyclonal antibodies with better specificity for tumor cells. The results from the preliminary immunological evaluation of these glycoconjugates in BALB/c mice indicate that these compounds lack immunogenicity and thus definitive conclusions cannot be drawn about the effect of extended antigen structures on the specificity of the immune response. Further immunological evaluation may require immunization experiments in a different strain of mice.

Acknowledgement

I am very grateful to my supervisor, Dr. David Bundle, for his support, patience, and guidance.

I would also like to thank Dr. Chang-Chun Ling for his advice and most helpful discussions--without him, none of this would have been possible. I thank Dr. O. Hindsgaul and his group members (Dr. B. Zhang and B. Rempel) for advising me in FAC/MS techniques and allowing me to use the FAC/MS instrument, equipment, and reference compounds; Dr. M. Palcic and group members for the donation of β-gal-1,4-transferase and instruction in performing enzymatic reactions (related to work not described in this thesis); Dr. I Pastan (from NIH) for the gift of monoclonal antibody B3; J. Sadowska for performing all the ELISA experiments and immunological assays; Dr. R. Whittal and Dr. A. Morales-Izquierdo for acquiring the mass spectra of my synthetic compounds, and Dr. P. Zhang, Dr. S. Andersen, and C. Nycholat for generously synthesizing and providing me with two acceptors.

I would also like to express my appreciation for all the Bundle group members, past and present, for their assistance and support over the years. In particular, the highly enjoyable, daily coffee breaks with Bernd Becker, Shirley Wacowich-Sgarbi, Henry Yu, Soren Andersen, Stephane Chambert, and Corwin Nycholat will not be forgotten.

Finally, I thank the following people for their patience, understanding, encouragement, and helpful discussions during my studies here in Edmonton: Jenny Liu, Lynn Liu, Marcus Liu, William Liu, Elaine Hung, Leah Terry, Joanne Yoon, Kai Brendel, Peggy Daley, Jenn Doubt, Grace Greidanus-Strom, Jennifer van Wijngaarden, Rob Bujalski, Michel Gravel, Bernd Keller, Chris Lovallo, Dave Manku, Hans Osthoff, and Steve Trepanier.

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List of Abbreviations

AAPH	2,2'-azobis(2-methylpropionamidine) dihydrochloride
Ac	acetyl
AIBN	2,2'-azobisisobutyronitrile
All	allyl
APT	attached proton test
Ar	aryl
Bn	benzyl
BSA	bovine serum albumin
Bt	column binding capactiy
Bz	benzoyl
c	concentration (g/100 mL)
C_8	octylsilane
C_{18}	octadecylsilane
CD1	first cluster of differentiation
COSY	correlation spectroscopy
CPG-SA	controlled pore glass-streptavidin
CPK	Corey-Pauling-Koltun
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMF	*N*,*N*-dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ES	electrospray ionization

Et	ethyl
FAC/MS	frontal affinity chromatography coupled online to mass spectrometry
Fmoc	fluorenylmethoxycarbonyl
Fuc	L-fucose
Gal	D-galactose
GalNAc	2-acetamido-2-deoxy-D-galactose
Glc	D-glucose
GlcNAc	2-acetamido-2-deoxy-D-glucose
h	hour
HHb	horse hemoglobin
HMQC	heteronuclear multiple quantum coherence
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
IC_{50}	concentration giving 50% inhibition
Ig	immunoglobulin
K_d	dissociation constant
K_d^{mix}	apparent K_d (measured for a ligand in the presence of a mixture of other ligands)
K_I	inhibition constant
KLH	keyhole limpet hemocyanin
Le	Lewis determinant
Le^b	Lewis B

Lev	levulinoyl
Le^x	Lewis X
Le^y	Lewis Y
MAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
Man	mannose
mBn	methoxybenzyl
Me	methyl
MHC	major histocompatibility complex
MS	molecular sieves or mass spectrometry
MWCO	molecular weight cutoff
m/z	mass-to-charge ratio
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
Pent	pent-4-enyl
Ph	phenyl
Phth	phthaloyl
ppm	parts per million
py	pyridine
RT	room temperature

SE	2-(trimethylsilyl)ethyl
Ser	serine
SIM	selected ion monitoring
TBDPS	*tert*-butyldiphenylsilyl
TCA	tumor-associated carbohydrate antigen
TCP	tetrachlorophthaloyl
TDS	thexyldimethylsilyl
TF	Thomsen-Friedenreich antigen
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TLC	thin-layer chromatography
TMB	3, 3', 5, 5'-tetramethylbenzidine
TMSOTf	trimethylsilyl trifluoromethanesulfonate
TOCSY	totally correlated spectroscopy
Tol	toluyl
Troc	trichloroethoxycarbonyl
TROESY	transverse rotating-frame Overhauser effect spectroscopy
UV	ultraviolet
$V\text{-}V_o$	break-through volume due to specific interactions
V_R	break-through volume due to specific interactions
Z	benzyloxycarbonyl

Chapter 1

Carbohydrate Conjugate Vaccines for Cancer Immunotherapy

1.1. Tumor-associated Carbohydrate Antigens

All cells have carbohydrates on their surface, conjugated to protein or lipids. These carbohydrates have diverse functions, such as contributing to structural integrity, masking the cell, or serving as receptors and ligands in recognition events.[1]

Many specific binding events between proteins and carbohydrates, as well as a few between carbohydrates, have been identified. There are three classes of proteins that bind carbohydrates: antibodies, enzymes, and lectins. Our understanding of protein-carbohydrate recognition, in particular those involving lectins,[2] is more advanced than our knowledge of carbohydrate-carbohydrate[3] recognition.

Results suggest that some carbohydrate recognition events mediate cell-cell, cell-matrix, cell-pathogen, and cell-substrate interactions. It has been proposed that carbohydrate-protein recognition accounts for the critical, initial specificity for the association, while other non-specific protein-protein interactions lend strength to the association.[3]

In most cases the biological functions of glycosylation and of specific recognition events involving sugars still remain to be deciphered; in contrast, the structures involved are often defined. Although much heterogeneity occurs in glycan chains, many terminal carbohydrate motifs have been identified, and it is their expression and distribution over cell surfaces that often serve as characteristic cell markers.

All tumor cells are aberrantly glycosylated on the surface of their cell membranes and therefore are distinct from their normal counterparts, i.e. normal cells of identical tissue type and stage of development.[4] The aberration may be a function of several factors: expression of carbohydrate antigens not found on the normal counterpart, over- or under-expression of normal carbohydrate antigens, and novel distribution of antigens on the surface of the cells.

The tumor-associated carbohydrate antigens (TCAs) are carbohydrate motifs often over-expressed on tumor cells (Figure 1.1). A tumor cell may synthesize multiple TCAs. Some of the TCAs, e.g. the Lewis antigens, are genetically and biochemically related to the blood group antigens (Figure 1.2). The blood group-related TCAs are characteristic of most epithelial tumors whereas ganglioside TCAs are characteristic of most neural crest-derived tumors.[5] The TCAs on tumor cells may mediate their aberrant behaviour, such as tumor growth, invasiveness, and metastasis, possibly via cell adhesion events.[4]

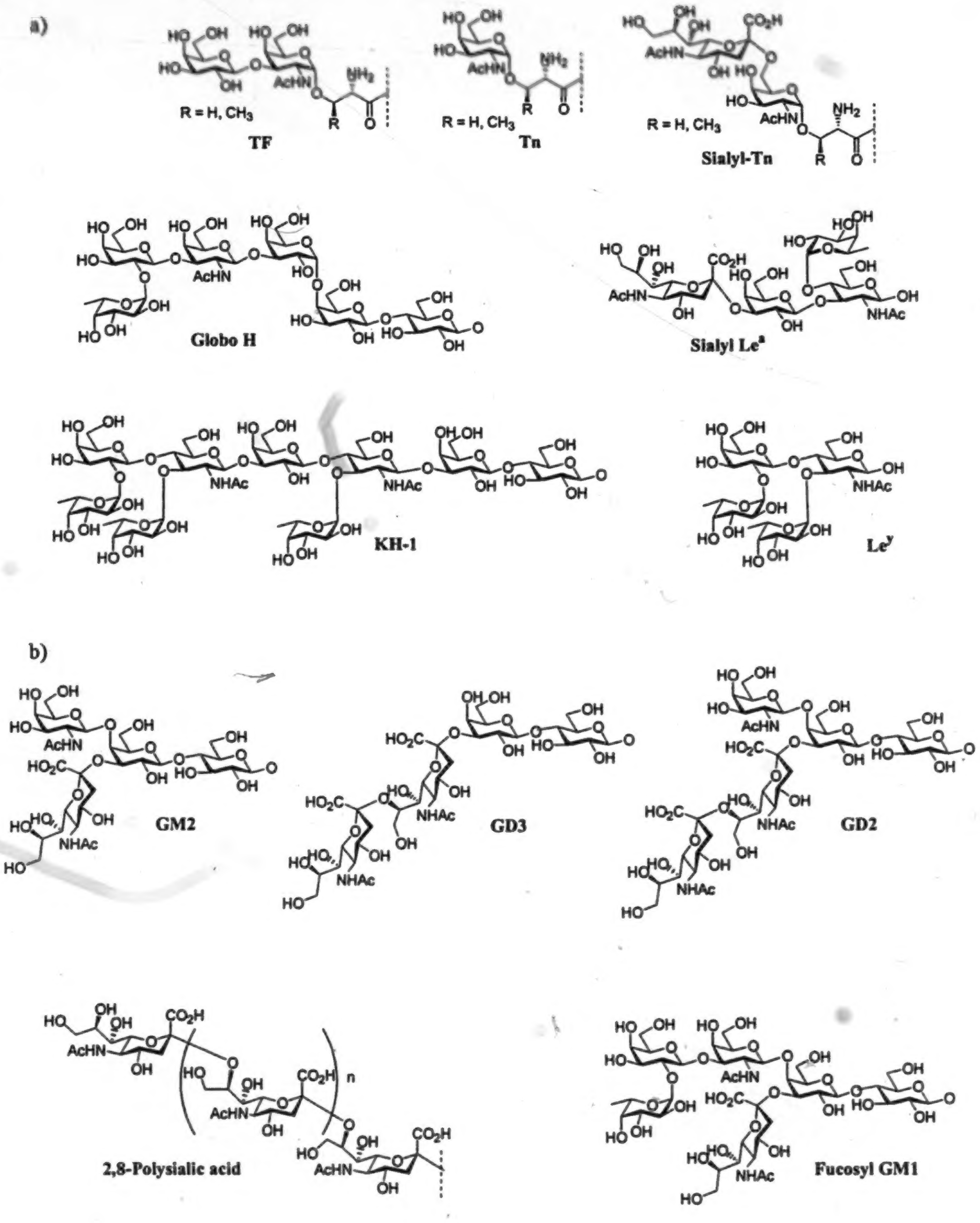

a)
R = H, CH3
TF
R = H, CH3
Tn
R = H, CH3
Sialyl-Tn
Globo H
Sialyl Le^a
KH-1
Le^y
b)
GM2
GD3
GD2
2,8-Polysialic acid
Fucosyl GM1

Figure 1.1 *Examples of a) blood group-related and b) ganglioside TCAs*

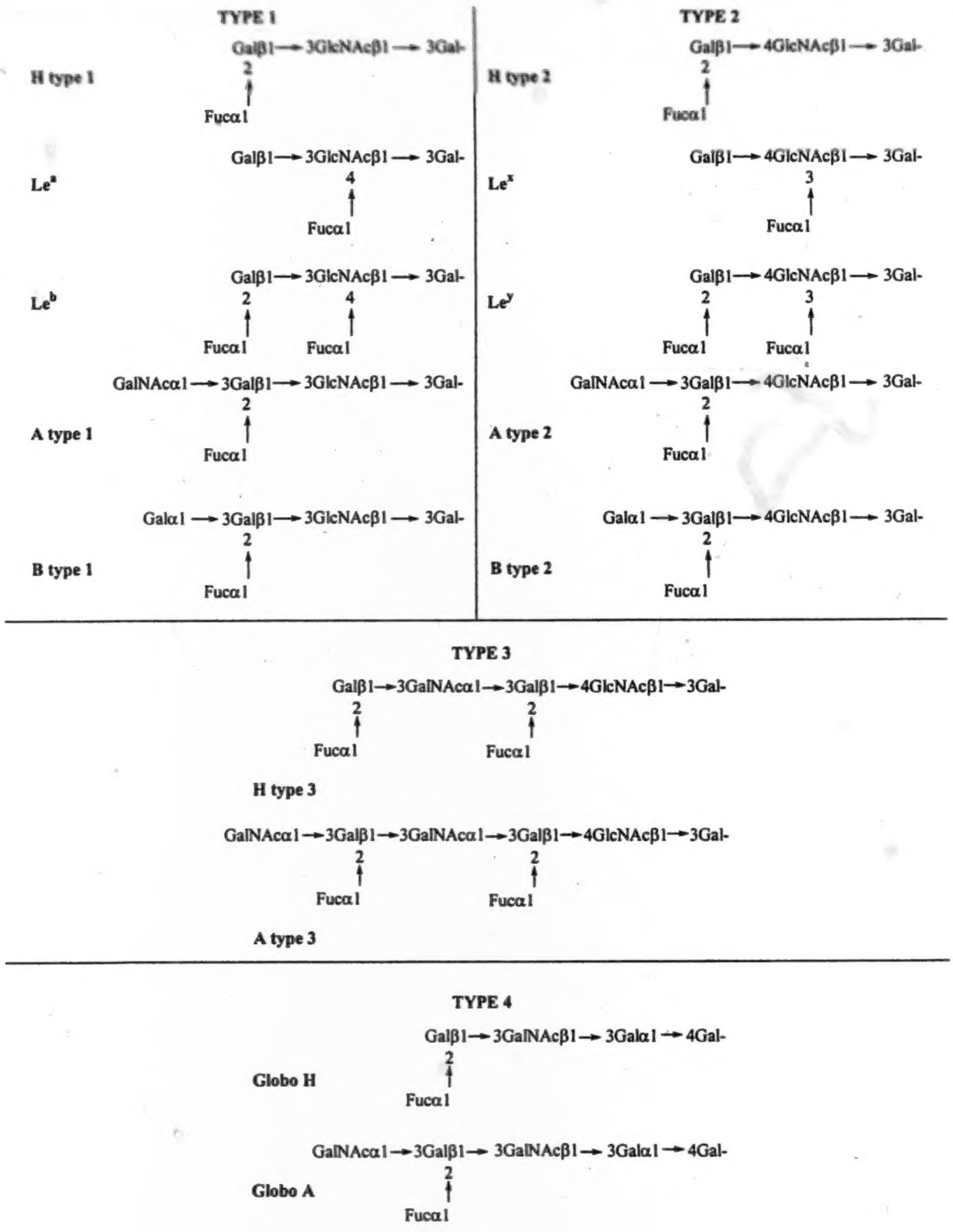

TYPE 1
H type 1
Galβ1→3GlcNAcβ1→3Gal-
2
Fucα1
Le^a
Galβ1→3GlcNAcβ1→3Gal-
4
Fucα1
Le^b
Galβ1→3GlcNAcβ1→3Gal-
2
4
Fucα1
Fucα1
A type 1
GalNAcα1→3Galβ1→3GlcNAcβ1→3Gal-
2
Fucα1
B type 1
Galα1→3Galβ1→3GlcNAcβ1→3Gal-
2
Fucα1
TYPE 2
H type 2
Galβ1→4GlcNAcβ1→3Gal-
2
Fucα1
Le^x
Galβ1→4GlcNAcβ1→3Gal-
3
Fucα1
Le^y
Galβ1→4GlcNAcβ1→3Gal-
2
3
Fucα1
Fucα1
A type 2
GalNAcα1→3Galβ1→4GlcNAcβ1→3Gal-
2
Fucα1
B type 2
Galα1→3Galβ1→4GlcNAcβ1→3Gal-
2
Fucα1
TYPE 3
Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Gal-
2
2
Fucα1
Fucα1
H type 3
GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Gal-
2
2
Fucα1
Fucα1
A type 3
TYPE 4
Globo H
Galβ1→3GalNAcβ1→3Galα1→4Gal-
2
Fucα1
Globo A
GalNAcα1→3Galβ1→3GalNAcβ1→3Galα1→4Gal-
2
Fucα1

Figure 1.2. *Structures of blood group A, B, H, and Lewis antigens*

1.2. Carbohydrate Conjugate Vaccines

Carbohydrates found on the outer leaflet of cell membranes make attractive targets for vaccines. Their location on the cell surface renders them accessible to the immune system. In addition, their expression on a cell surface may be both peculiar to, and characteristic of, a cell type in its specific state of health or disease. These two facts suggest it should be possible to exploit carbohydrate motifs as specific cell markers.

There is, however, a significant disadvantage to employing carbohydrate vaccines, and that is carbohydrates are generally poor immunogens. Unless they are microbial components, they must usually be conjugated to other biomolecules, most often proteins, in order to elicit a humoral immune response, and even then, the affinity of the antibody generated is generally lower than that of an antibody that binds a protein antigen.[6]

The dependence on conjugation for carbohydrate immunogenicity stems from the process of B cell activation.[7, 8] B cell activation is essential to the humoral immune response, for it is the B cells that produce the molecules that mediate specific immunity (i.e., antibodies). In general, there are three signals that are necessary for B cell activation (Figure 1.3), and most carbohydrates intrinsically may trigger only one of these.

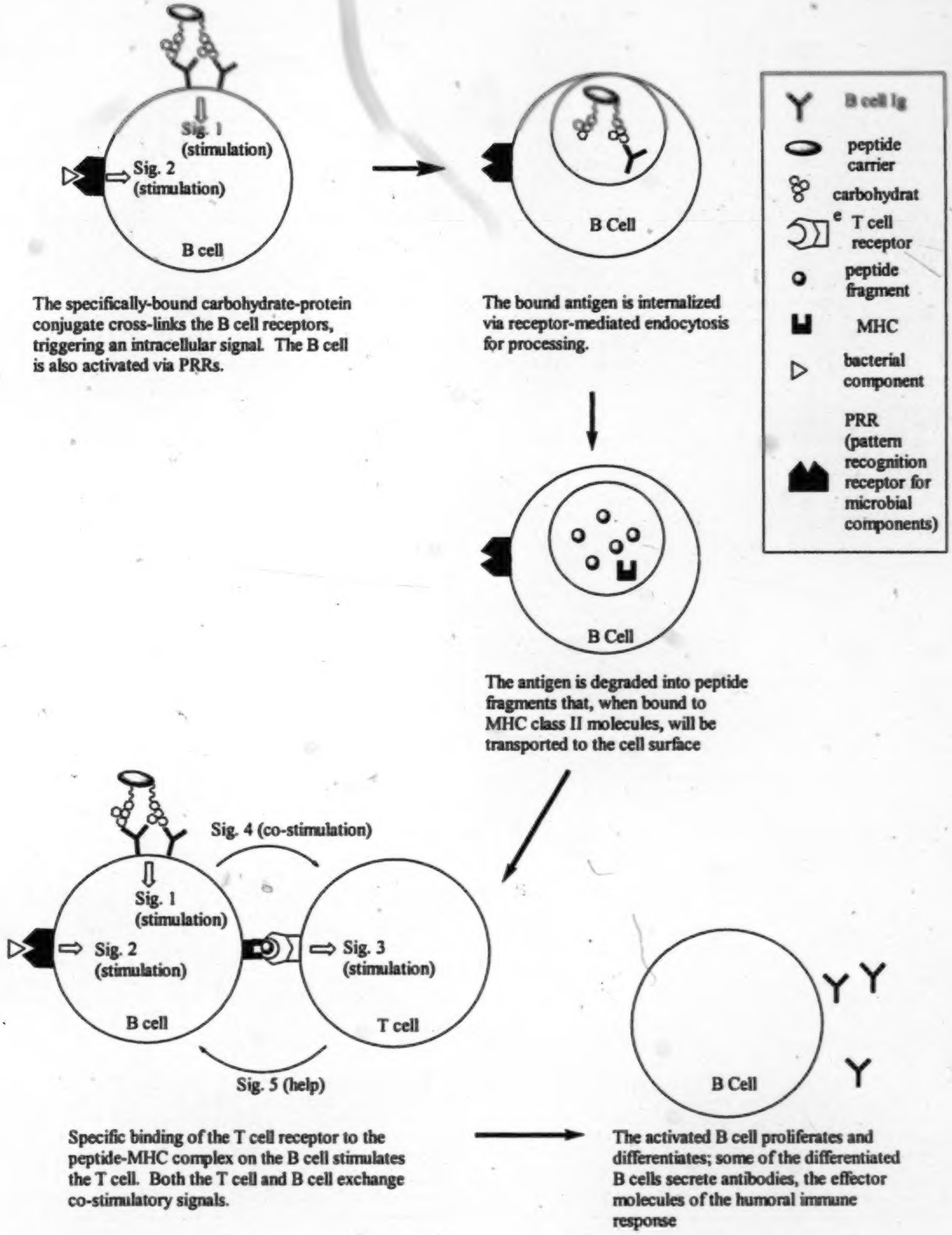
One event necessary for B cell activation is the specific binding of antigen to the B cell's membrane-bound immunoglobulin (Ig) receptors. If such binding leads to cross-linking of the receptor molecules, the aggregation of receptors in turn leads to an intracellular signalling cascade.

The second event also often necessary for B cell activation is the engagement of a helper T cell. This happens after the B cell internalizes the specific antigen bound to its receptors by receptor-mediated endocytosis and degrades the antigen to generate peptide fragments. The B cell loads the exogenous peptide fragment into a MHC class II molecule. The peptide-MHC complexes are then displayed on the surface of the B cell where only a helper T-cell with a receptor that recognizes both the peptide fragment and the MHC molecule will bind. If there are microbial particles about (as from a bacterial adjuvant introduced simultaneously with the antigen), the B cell will be capable of stimulating a T cell that binds specifically to the peptide-MHC complex on the B cell. The T cell, stimulated both by the B cell and by recognition of the MHC complex, will then release molecules that serve as stimulatory signals to the B cell.

Therefore, the signals that stem from antigen and microbial binding to surface receptors as well as the signals released by the helper T cell serve to activate the B cell. Once activated, the B cell proliferates. Some of the B cell progeny become plasma cells and secrete immunoglobulins of different classes and functions while others become memory cells.

Carbohydrate antigens may bind to B cell receptors, and thus may generate one of the stimulatory signals required for B cell activation. However, in order to obtain T cell help, the B cell must present to the helper T cell a foreign antigen appropriately packaged in a self-molecule (the MHC), and it is here that carbohydrates fail as immunogens. Although there may be mechanisms as yet undiscovered, at present it appears that cells have no means of presenting purely carbohydrate antigens to T cells. That is, while a cell may present to a T cell either a peptide antigen bound to a MHC complex, or a glycolipid antigen bound to a CD1 complex,[9-11] it does not synthesize a molecule that will bind and display a carbohydrate.

Nonetheless, if appropriately conjugated and administered with an appropriate adjuvant, carbohydrates are indeed capable of raising protective antibodies,[12] and the development of carbohydrate conjugate vaccines is an area of considerable research interest.


Sig. 1
(stimulation)
Sig. 2
(stimulation)
B cell
B Cell
B Cell
Sig. 4 (co-stimulation)
Sig. 1
(stimulation)
Sig. 2
(stimulation)
Sig. 3
(stimulation)
B cell
T cell
Sig. 5 (help)
B Cell
B cell Ig
peptide carrier
carbohydrate
T cell receptor
peptide fragment
MHC
bacterial component
PRR (pattern recognition receptor for microbial components)

The specifically-bound carbohydrate-protein conjugate cross-links the B cell receptors, triggering an intracellular signal. The B cell is also activated via PRRs.

The bound antigen is internalized via receptor-mediated endocytosis for processing.

The antigen is degraded into peptide fragments that, when bound to MHC class II molecules, will be transported to the cell surface

Specific binding of the T cell receptor to the peptide-MHC complex on the B cell stimulates the T cell. Both the T cell and B cell exchange co-stimulatory signals.

The activated B cell proliferates and differentiates; some of the differentiated B cells secrete antibodies, the effector molecules of the humoral immune response

Figure 1.3. *Protein antigens conjugated to carbohydrate antigens allow T cells to help activate carbohydrate-specific B cells*

1.3. Focusing on Tumor-associated Carbohydrate Antigens for Cancer Immunotherapy

Vaccines are used to induce protective immunity and have traditionally been administered for prophylactic purposes. Employed in this way, they help to pre-equip the immune system to attack pathogens that intrinsically elicit only a poor immune response, or to rapidly neutralize a pathogen and thereby reduce the damage it would otherwise cause in the time it would take to mount a primary immune response.

The ultimate goal of cancer vaccines would be to actively induce immunity to cancer in humans by breaking the tolerance the immune system has for cancer.

Using the body's own immune system to reject tumors would be the ideal therapy for cancer: it would be specific, operational throughout the body, and the long-term memory would prevent metastasis and recurrence.[13] Alternatively, cancer vaccines could be used to generate antibodies to passively protect the patient.[14] These anti-tumor antibodies, if conjugated to other substrates, could also be useful as diagnostic aids, or as carriers for targeting drugs[15, 16] and radioisotopes[17] selectively to tumor cells.

The necessity for cancer vaccines stems from the low immunogenicity of most cancers. Although cancer cells are abnormally glycosylated and therefore might be expected to appear "foreign" to the immune system, there are a number of means by which tumor cells evade detection by the immune system and are tolerated.[7, 8, 18]

One reason for the low immunogenicity of tumors is that many TCAs are autoantigens, expressed on some normal tissues, and the immune system generally tolerates these. Further, these TCAs are often surrounded by autoantigens, which decreases their immunogenicity; the immune system responds more strongly to antigens presented in a foreign context than to antigens presented in a more familiar context.

There is also the possibility that the tumor cells lack antigens altogether. They may produce only autoantigens, may lose their antigens by mutation and genetic instability, or may have their antigens destroyed by the immune system itself. The last case might arise if antibody binding to a TCA effects degradation of the TCA only, and not to elimination of the tumor cell, leading to the selection of mutant tumor cells that do not express the TCA.

Even with sufficient antigens on their surface, tumor cells still avoid rejection by the immune system. Some do so by synthesizing immunosuppressive molecules. Alternatively, they may mutate and thereby stop synthesizing the molecules that would be required for stimulating a T cell response, e.g. MHC molecules for presenting their abnormal antigens to the T cells.

The rationale for carbohydrate-based cancer vaccines[14] is this: conjugate vaccines that display TCAs in an immunogenic context should raise anti-TCA antibodies. These antibodies that execute the humoral response against the conjugate vaccine should also cross-react with the natural TCAs on tumor cells.

Lewis Y (Le^y) is a TCA that has been incorporated into several conjugate vaccines.[19, 20] Le^y is expressed on a majority (more than seventy percent)[21] of epithelial cancers, including breast, gastrointestinal, non-small-cell lung, cervix, ovary, and some melanomas. Normal tissues, mostly of the gastro-intestinal tract,[22] also express Le^y, but at lower levels. The combination of its high frequency and dense expression on a majority of cells of many types of cancers (e.g., 80% of all breast cancers,[17] ca. 2×10^5 copies per cell[15]) with its limited expression on normal cells accounts for the popularity of Le^y as a target for carbohydrate-based cancer vaccines. The Le^y antigen has the added merit of being rapidly internalized upon ligand binding, thus rendering it an attractive target for antibody-conjugated drugs as well.[16, 23]

Examples of other TCAs targeted by conjugate cancer vaccines are GM2,[24] globo H,[25, 26] Tn,[19] and sialyl-Tn.[13, 27] The research in this area has shown some promise for cancer vaccines,[28] where the presence of anti-TCA antibodies in patients correlates with a more favourable prognosis.[18]

1.4. Probing the Relationship between Immunogen Structure and Antibody Specificity

1.4.a. Context-dependent Antibody Recognition: Exploring Antigen Size

B3 is an IgG1κ monoclonal antibody (mAb) that was isolated after immunization of mice with breast cancer cells. The epitope recognized by B3 was deduced from its cross-reactivity against a panel of synthetic glycoconjugates. It was determined that B3 recognizes the TCA Le^y as well as di- and trifucosyl Le^x.[29]

Immunocytochemistry experiments indicated that B3 reacted strongly with many cancer specimens and cell lines but not to most normal tissues, making it potentially useful for diagnostic and therapeutic purposes[17, 30] as well as for clarifying the basis of tumor selectivity.

Tumor-associated carbohydrate antigens are also autoantigens expressed to some degree on normal cells. Some antibodies that have been found to react only to TCAs on cancers and not to those on normal cells may be capable of such selectivity by recognizing their antigens in a density-dependent manner. On cancer cell membranes, the TCAs are often over-expressed and may cluster to form glycolipid microdomains.[31] An antibody that has a low affinity for an antigen may not bind if the antigen is expressed at levels below a certain threshold[29] and may therefore exhibit tissue selectivity.

It is also possible that some anti-TCA antibodies are capable of tumor selectivity by recognizing multivalent clusters of glycolipids through self-association into antibody clusters. In some cases, antibodies are able to self-associate through their Fc domains, as is the case for murine IgG3. There is also the possibility of self-association through the Fab domain; such antibodies that are capable of this "homophilic binding" have two idiotopes: one for recognition of the TCA and one for dimerization.[6]

Alternatively, the selectivity of some antibodies for TCAs on only tumor cell membranes may be due to their ability to recognize extended structures of TCA antigens, since carcinoma cells have the ability to synthesize extended type 2 chain antigens.[32]

Using the crystal structure of anti-Le^y mAb BR96 complexed with Le^y,[33] Blaszczyk-Thurin and co-workers compared the sequence and structural properties of several anti-Le^y antibodies: BR55-2, B3, H18A, and BR96.[32] Their results indicated that BR96, BR55-2, and B3 all interact with the Le^y tetrasaccharide in a similar fashion. Furthermore, they observed from the analysis of their model that BR55-2, like BR96, may accommodate an extended Le^y structure with an additional trisaccharide (fucosylated lactosamine) at the reducing end. The putative cross-reactivity of these mAbs with the extended structures was not tested, however. We would like to explore the possibility of extended structure recognition in B3 by determining its binding affinity for synthetic Le^y analogs.

1.4.b. Exploring the Effect of Employing Larger Structures for Immunization

The greater challenge in developing carbohydrate vaccines lies not so much in the synthesis, but in the rational design. The general guidelines for the structural requirements of an optimal vaccine have not been established in a systematic fashion.[34]

One general approach to the design of a vaccine is to deduce the binding specificity of protective antibodies raised against natural antigens in order to define the immunodominant structural motif involved in recognition and, having identified the minimal epitope, to proceed on the assumption that a structure that satisfies the minimal binding requirements is also a suitable structure for immunization purposes.[35, 36] Similarly, there is research devoted to carbohydrate mimicry using peptides, since amino acids are simpler, more tractable chemical building blocks than sugars.[37, 38] The purpose is to reduce the effort required to chemically synthesize the vaccines by using simpler, truncated structural mimics than the carbohydrate antigen itself. This approach leads to the employment of immunizing structures that are optimized for binding to a pre-existing antibody site that recognizes some natural epitope, but that are not necessarily optimized for generating these antibody binding sites.

While the approach of immunizing with minimal structures has its merits and has been shown to be successful,[36] it would still be preferable to know what the ideal structure would be so that one can compromise and design vaccines that balance synthetic accessibility with effective immunogenicity.

The presentation of the same primary carbohydrate structure in different vaccines can greatly affect the immune response. For example, Kitamura and co-workers found significant differences between mAbs raised against synthetic Le^y neoglycoproteins and mAbs raised against Le^y positive human tumor lines.[21] Anti-synthetic Le^y mAbs, in addition to being of different isotypes, reacted poorly with natural Le^y and showed greater cross-reactivity with Le^x or H-type 2 structures. In contrast, anti-tumor mAbs reacted with both natural and synthetic Le^y and showed less cross-reactivity with related structures.

It may be prudent to immunize with a larger structure than simply the antigen of interest for a couple of reasons. The natural antigens targeted are generally found at the terminus of larger structures, so an antibody that is expected to bind to the natural antigen may have to accommodate extra residues.[39] An antibody that is generated against the minimal structure may not be able to do so. Further, it is possible that the accommodation of additional residues at the non-reducing end may result in greater tumor selectivity in the antibodies raised, if the peripheral residues on the carbohydrate antigen presented on the target cell differ from those on other normal cells presenting the same primary carbohydrate motif.

With our research, we aim to advance our understanding of the immune system with respect to the optimal size of the antigen on the immunogen. Our approach was to synthesize conjugate vaccines that carry two different extended Le^y structures to determine if these vaccines raise antibodies with better affinity for the natural antigen.

1.5. Scope of Projects

Described in the following chapters is research aimed at elucidating a general relationship between the immunogen structure and the resultant antibody paratope.

The synthesis of three Le^y analogs of varying size and the assay of their activity with anti-tumor mAb B3 is reported first. Estimates of the binding affinity for the three Le^y analogs were obtained via frontal affinity chromatography coupled to mass spectrometry (FAC/MS) and are supported by ELISA experiments. These studies were carried out to explore the possibility that extended structure recognition is the origin of B3's tumor specificity.

The synthesis and immunological evaluation of two Le^y-based vaccine candidates that mimic the natural context of the antigen is discussed next. Unlike previously reported Le^y-based anti-cancer vaccines, the vaccine candidates described here present the Le^y antigen in the context of a glycolipid. These experiments were aimed at probing the hypothesis that higher titers of tumor-specific antibodies will be elicited by immunization with extended structures that more closely mimic the natural antigen.

Chapter 2

Synthesis of Lewis Y Analogs as Ligands for Monoclonal Antibody B3

2.1. Synthetic Compounds to Assay B3 Reactivity

We synthesized three Le^y derivatives (Figure 2.1) to investigate the possibility that anti-tumour monoclonal antibodies such as B3 recognize extended Le^y structures. Two of the synthetic structures (**2** and **3**) mimic the presentation of Le^y in the context of the natural glycolipid antigen by incorporating additional sugars that would generally be found at the reducing end of the Le^y determinant.

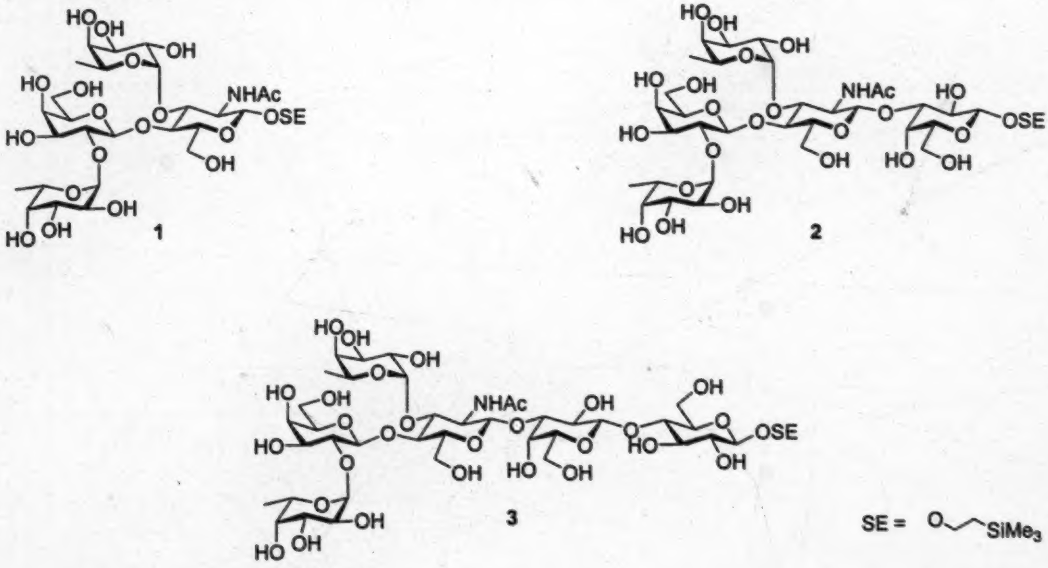


Figure 2.1. *Lewis Y analogs synthesized as ligands for monoclonal antibody B3*

2.2. Background

2.2.a. Challenges and Strategies in Oligosaccharide Synthesis

Since most of the monosaccharides that constitute mammalian complex oligosaccharides are commercially available, the major challenge in oligosaccharide synthesis lies in the formation of glycosidic bonds with controlled stereoselectivity and chemoselectivity.

To control the stereochemistry of the glycosidic bond between a donor and an acceptor, one may alter the anomeric leaving group on the donor, the substituents on the donor, the promoter, the solvent, the temperature,[40] and the orientation of the hydroxyl on the acceptor.[41] One may also use auxiliary tethers for intramolecular delivery of the aglycon.[42]

The problem of regioselectivity arises from each monosaccharide possessing multiple functional groups (usually amine and hydroxyl moieties) of similar reactivity. There is indeed a range in reactivity, depending on the position of the hydroxyl and the configuration of the pyranose (e.g. for the $^4C_1$ ring conformation of glucopyranoside, the order of reactivity[40] is 6-OH >> 3-OH > 2-OH > 4-OH), but usually the differences are not sufficiently large to ensure selectivity. Glycosylation at the desired site is usually effected with protective groups: in general, one reactive hydroxyl on the acceptor is left free while the other nucleophilic groups on both the acceptor and donor are blocked.

Unfortunately, the use of protective groups brings its own challenges to the synthesis of oligosaccharides. The protective group strategy must ensure orthogonality and compatibility of all functional groups throughout the synthetic sequence. Further, installation of differential protection at the sugar hydroxyls is often a multi-step process, and the conclusion of a synthesis may require several steps to cleave the orthogonal protective groups.

Even if the protecting groups are chemically inert in the synthetic sequence, they affect the properties (e.g. solubility and crystallinity[43]) of the sugar. They also have an influence on the reactivity of the parent carbohydrate through steric,[41] electronic,[44] and torsional effects.[45] Since small changes in the substituents on a pyranose can have a large influence on its reactivity, the successful, stereocontrolled formation of a glycosidic bond depends on the structure and reactivity of both the donor and the acceptor. The importance of the compatibility ("matching"[41]) of the stereoelectronic properties of a donor and acceptor pair makes each new oligosaccharide target a new problem to be solved.

Although protective group effects add complexity to the problem of oligosaccharide synthesis, they may at the same time be exploited in glycosylation strategies.[46] With substituents "tuning" the reactivity of the donor and the acceptors, chemoselectivity can be introduced to reactions where it would not otherwise be present.

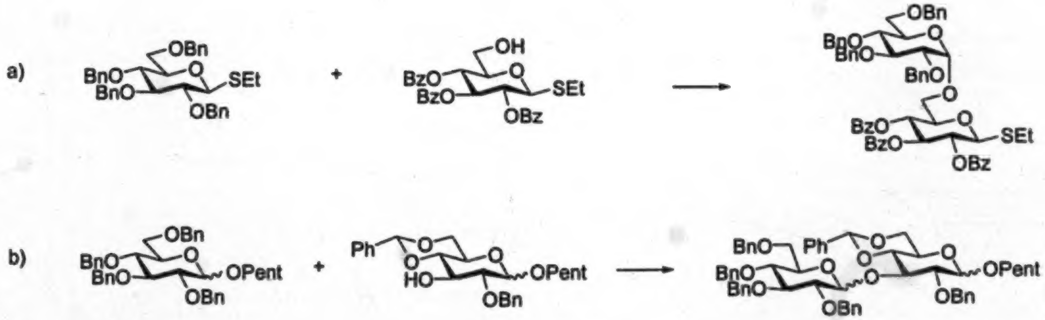


Figure 2.2. *Examples of chemoselective glycosylations controlled by protective groups.*[46] *Self-condensation does not occur. (a) Acyl substituents reduce the reactivity of a donor compared to alkyl groups. (b) The fused ring of a cyclic acetal reduces the reactivity of a donor by inflicting torsional strain upon the developing oxo-carbenium ion intermediate. In both A and B, the product itself is also a potential donor.*

Shown in Figure 2.2 are some examples of glycosylation strategies that rely on the effect of the protective groups, on both the donor and the acceptor, to dictate the selectivity of the reaction. In contrast, Figure 2.3 shows examples of strategies in which chemoselectivity arises not from the protective groups on the donor-acceptor combination, but from the nature of the anomeric leaving group of the donor.[46, 47] In all of these strategies, the product of the coupling reaction may be used as a donor, without further manipulation, in a subsequent glycosylation reaction.

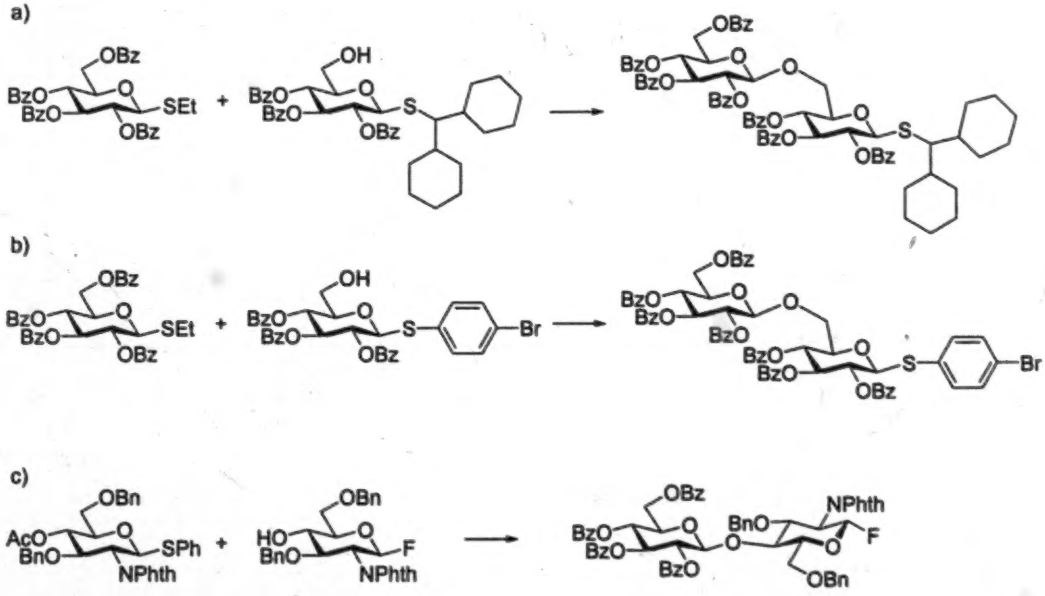


Figure 2.3. *Examples of chemoselective glycosylations controlled by modifying the anomeric leaving group.*[46, 47] *Self-condensation does not occur. (a) Bulky thiol aglycons reduce the reactivity of a donor. (b) Decreased electron-donating properties of thiol aglycons reduce the reactivity of a donor. (c) "Orthogonal glycosylation" results from selective activation of different anomeric leaving groups. In A, B, and C, the product itself is also a potential donor.*

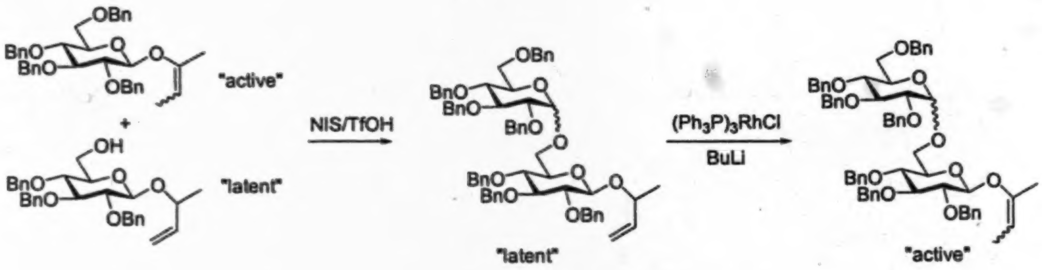


Figure 2.4. *"Latent-active glycosylation strategy", a specific example of a strategy in which the product is not a donor, but may be transformed into one by modification of the aglycon. Here isomerization of the allyl aglycon to the vinyl aglycon transforms a sugar from its "latent" (unreactive) state to its "active" (reactive) donor state.*

A different approach, less convergent than the others but still useful for block synthesis, is to couple a donor with an acceptor whose anomeric protective group can be transformed into a leaving group, under mild conditions, in one or two steps (Figure 2.4).[48] That is, in contrast to the aforementioned strategies, the product of the glycosylation reaction is not an immediate donor, but can be converted into one with ease.

Solid-phase synthesis,[49] reactivity-based one-pot glycosylation, and enzymatic synthesis[50] are three methods under development that may prove to be less problematic than traditional oligosaccharide synthesis. The first approach simplifies work-ups and product isolation, though not necessarily product purification. The second method reduces the number of purification steps required, and the last approach reduces the need for protective group manipulations. At present, the full benefits of these methods are still unrealized.

Solid-phase and one-pot glycosylation strategies do not eliminate protective groups and their associated problems. It is noteworthy that the differential protection of mono- and disaccharide building blocks consumes the most time and effort in oligosaccharide synthesis, not the coupling steps. Thus, the extent to which these alternative strategies can facilitate carbohydrate synthesis is still limited by the efficiency of the reactions used in traditional synthesis. In contrast, enzymatic synthesis would be ideal, but at present this approach is hampered by the availability and cost of the enzymes and their sugar substrates.

2.2.b. Previously Reported Syntheses of Le^y Analogs

The structure of the Le^y determinant was first described in 1966.[51] The tetrasaccharide Le^y determinant was also discovered to constitute a part of a different, larger carbohydrate determinant, the tumor-associated carbohydrate antigen KH-1 (Figure 1.1).[52] Although the structures of the blood group and related Lewis antigens had been known for some time, the first synthesis[53] of Le^y was described only in 1977. Since then, however, a number of alternative synthetic routes have been reported for both Lewis Y [54-66] and structures related to KH-1.[58, 67-70]

In some cases, Le^y has been an attractive target for synthesis simply to illustrate the scope of selected methodologies, such as one-pot glycosylation,[66] or synthesis on polymeric support.[65] Le^y analogs have also been synthesized to provide access to the compound in sufficient quantities for glycobiological studies, e.g. for incorporation into conjugate vaccines[19] or as a probe for lectin binding sites.[61, 62]

The retrosynthetic analysis of the Le^y determinant has been carried out in four different ways (Figure 2.5, 2.6, 2.7). The only feature common to all four approaches is the use of a single fucosyl donor for the installation of both fucose moieties.

Table 2.1 *Building blocks used in the synthesis of Le^{y} derivatives. The lactosamine moiety is derived either enzymatically[a] or from lactal[b]*

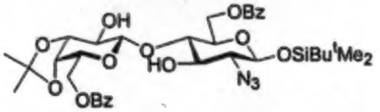
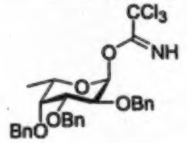
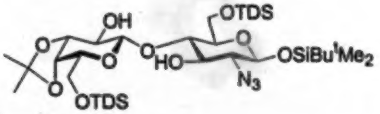
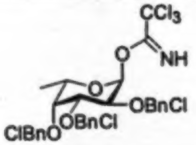
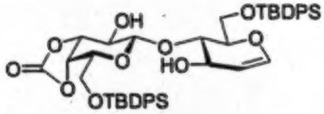
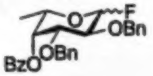
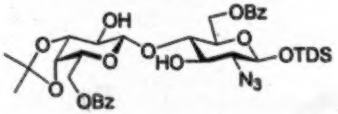
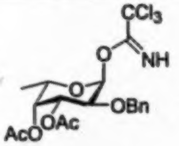
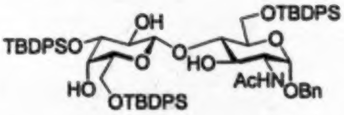
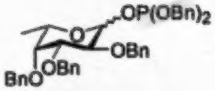
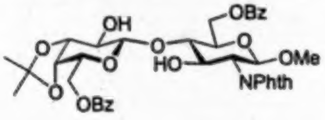
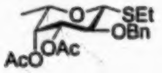
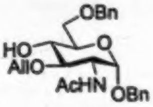
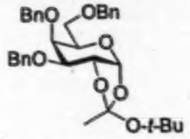
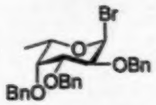
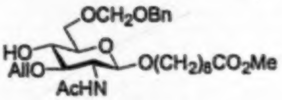
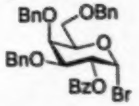
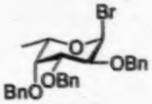
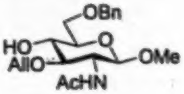
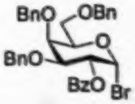
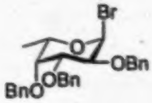
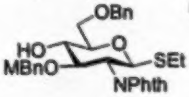
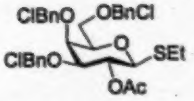
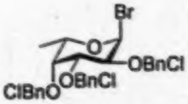
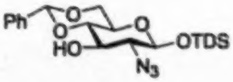
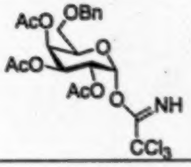
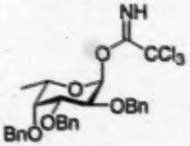
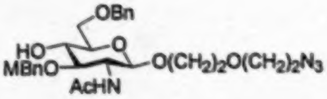
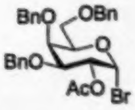
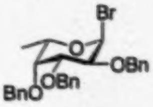
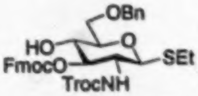
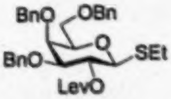
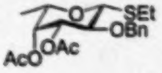
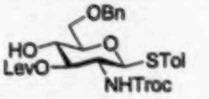
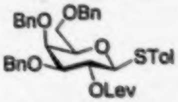
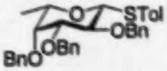
Entry	Lactosamine	Fucose	Ref.
1[b]	 OH OBz O O O HO OSiBu^{t}Me$_2$ N$_3$ O OBz	 CCl$_3$ NH O O OBn BnO OBn	54
2[b]	 OH OTDS O O O HO OSiBu^{t}Me$_2$ N$_3$ O OTDS	 CCl$_3$ NH O O OBnCl ClBnO OBnCl	55
3[b]	 OH OTBDPS O O= O HO O OTBDPS	 O F OBn BzO OBn	56, 57
4[b]	 OH OBz O O O HO OTDS N$_3$ O OBz	 CCl$_3$ NH O O OBn AcO OAc	58
5[a]	 OH OTBDPS TBDPSO O O HO O HO OTBDPS AcHN OBn	 OP(OBn)$_2$ O OBn BnO OBn	59
6[b]	 OH OBz O O O HO OMe NPhth O OBz	 O SEt OBn AcO OAc	60

Table 2.2. *Building blocks used in the synthesis of Le^y derivatives from monosaccharides*

Entry	Glucosamine	Galactose	Fucose	Ref.
1	 OBn HO AllO AcHN OBn	 BnO OBn BnO O-t-Bu	 Br OBn BnO OBn	53
2	 OCH₂OBn HO AllO O(CH₂)₈CO₂Me AcHN	 BnO OBn BnO BzO Br	 Br OBn BnO OBn	61
3	 OBn HO AllO OMe AcHN	 BnO OBn BnO BzO Br	 Br OBn BnO OBn	62
4	 OBn HO MBnO SEt NPhth	 ClBnO OBnCl ClBnO SEt OAc	 Br OBnCl ClBnO OBnCl	43
5	 Ph HO OTDS N₃	 AcO OBn AcO AcO NH CCl₃	 NH CCl₃ OBn BnO OBn	63
6	 OBn HO MBnO O(CH₂)₂O(CH₂)₂N₃ AcHN	 BnO OBn BnO AcO Br	 Br OBn BnO OBn	64
7	 OBn HO FmocO SEt TrocNH	 BnO OBn BnO SEt LevO	 SEt OBn AcO OAc	65
8	 OBn HO LevO STol NHTroc	 BnO OBn BnO STol OLev	 STol OBn BnO OBn	66

As may be seen from Tables 2.1 and 2.2, a variety of different fucose derivatives have been employed in the synthesis of Le^y. The enhancement of α-glycosidation is the major consideration in the selection of protective groups for this building block. α-Selectivity at the anomeric centre is best achieved by masking the 2-OH with a substituent that is incapable of neighbouring group participation during the glycosylation reaction; in this case, the universal choice is the formation of a benzyl ether. The chlorobenzyl ether used in a couple of instances may have been selected for its better crystallization properties[43] than the benzyl ether, or for ease of removal.[71]

The most common protective group pattern employed is 2,3,4-tri-*O*-benzylation. In the cases where the hydroxyls are differentially protected, the acyl substituents at the O-3 and O-4 were employed to decrease the reactivity of the fucosyl donor and thereby increase α-selectivity in glycosidation.

Figure 2.5 shows one retrosynthetic strategy adopted for the synthesis of the Le^y determinant: the glycosidic linkage between each hexose is disconnected in three separate steps. Although relatively linear in its approach, this scheme reduces the number of protective group manipulations required to differentially protect the glucosamine building block, since early installation of the fucose moiety on the 3-OH of glucosamine blocks that position. Also beneficial is the masking of the 2-amino functionality on glucosamine as an azido group, which enhances the reactivity of the 4-OH towards galactosylation.[72] One potential drawback to this scheme is that, occasionally, attempts to galactosylate the 4-OH of glucosamine when the 3-OH is fucosylated fail.[73]

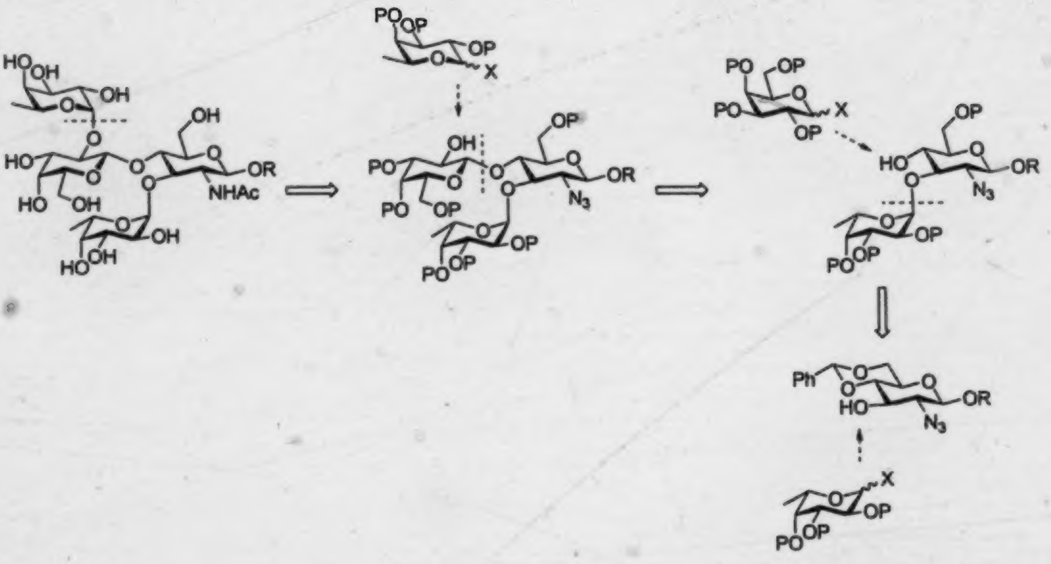

HO OH
OH
O
HO
O
O
OH
O
OR
NHAc
HO OH
O
OH
HO OH
PO OP
OP
O
X
OH
PO
O
OP
O
OR
N3
PO OP
O
OP
PO OP
PO OP
O
X
PO
OP
OP
HO
O
OR
N3
O
OP
PO OP
Ph
O
O
O
HO
OR
N3
X
O
OP
PO OP

Figure 2.5. *Linear Le^y retrosynthetic strategy. The glycosidic linkages are disconnected sequentially: fucα1→ 2, galβ1→ 4, fucα1→ 3. P = protective group; X = anomeric leaving group. (See Table 2.2, entry 5).*

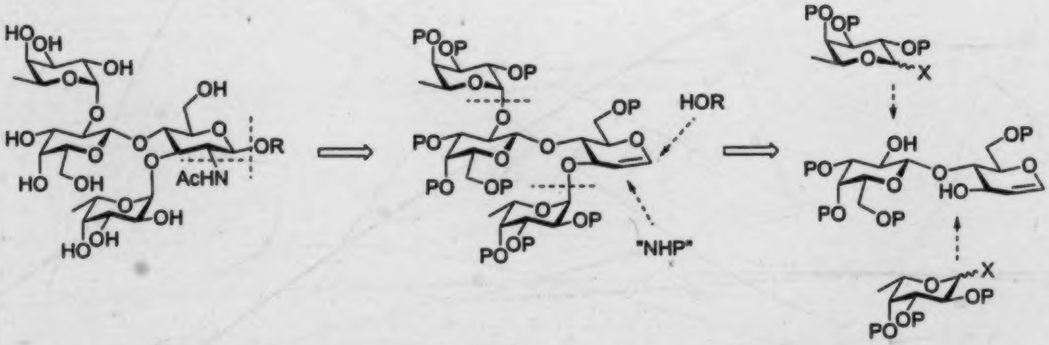

HO OH
OH
O
HO
O
O
OH
O
OR
AcHN
HO OH
O
OH
HO OH
PO OP
OP
O
PO
O
OP
O
PO OP
O
OP
PO OP
HOR
"NHP"
PO OP
OP
X
OH
PO
O
OP
O
HO
PO OP
X
O
OP
PO OP

Figure 2.6. *Le^y retrosynthetic strategy in which the 2-amino and aglycon moieties are disconnected first to give a glycal precursor. The tetrasaccharide glycal is further disconnected to give lactal and a fucosyl donor. P = protective group; X = anomeric leaving group. (See Table 2.1, entry 3)*

A shorter retrosynthetic scheme (Figure 2.6) first disconnects the aglycon and the 2-amino substituent from the Le^y determinant. The tetrasaccharide is then further disconnected at both fucosidic bonds simultaneously into a fucose donor and a lactal acceptor.

This scheme shortens the synthetic sequence leading to the protected tetrasaccharide by minimizing the number of glycosylations and simplifying the protective group strategy. However, it requires additional manipulations, including installation of the 2-acetamido functionality and the aglycon, late in the synthesis at the tetrasaccharide stage.

The two most convergent retrosynthetic strategies to the Le^y determinant are shown in Figure 2.7 (A and B). The two fucosidic linkages of the tetrasaccharide are disconnected simultaneously; in this case the precursors are a fucose donor and a lactosamine acceptor. The lactosamine moiety may be synthesized either from lactal (route A, Figure 2.7) or from a galactose donor and glucosamine acceptor (route B, Figure 2.7).

In contrast to the scheme in Figure 2.6, which also invokes a lactal building block, in route A (Figure 2.7) the 2-amino functionality, masked as an azide, is introduced prior to fucosylation. This route thereby reduces the number of manipulations at the tetrasaccharide stage while still taking advantage of the $\beta(1\rightarrow 4)$ glycosidic linkage pre-installed in lactal.

The retrosynthetic strategy in route B (Figure 2.7) was employed in the first synthesis[53] of Le^y, as well as the most recent,[66] and is the most commonly adopted approach.

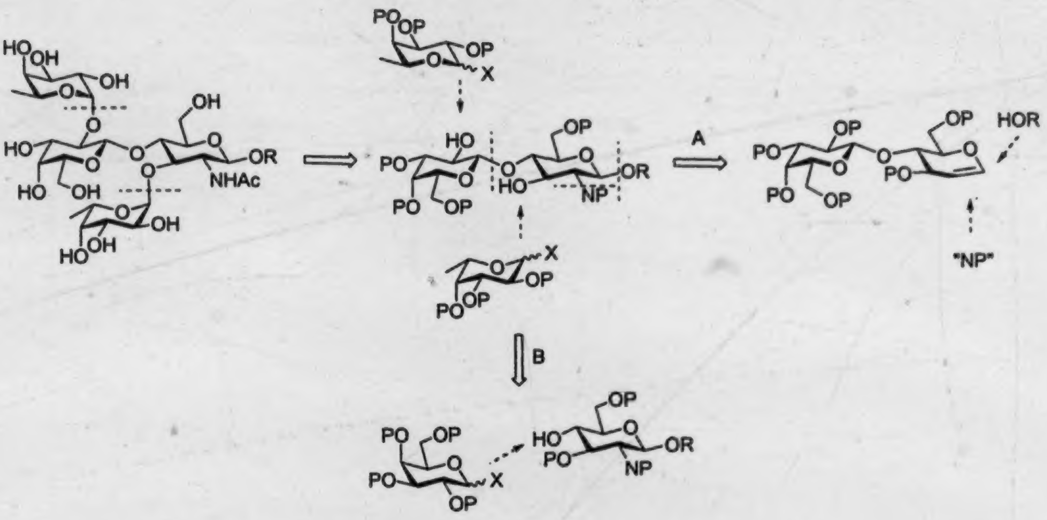


Figure 2.7. *Le^y retrosynthetic strategies in which two disconnections are made simultaneously to give a fucose donor and lactosamine acceptor. P = protective group; X = anomeric leaving group. Route A: lactosamine is derived from lactal (see Table 2.1, entries 1, 2, 4 and 6). Route B: lactosamine is constructed from galactose and glucosamine, either enzymatically (see Table 2.1, entry 5) or chemically (see Table 2.2, entries 1-4, 6-8).*

The disadvantage to constructing the lactosamine moiety from two monosaccharide building blocks (route B) instead of using a lactal precursor (route A) is that the synthetic sequence leading up to the diol acceptor is lengthened. Formation of the $\beta(1\rightarrow 4)$ linkage necessitates not only one more glycosylation step, but also several reactions to install differential protective groups on the galactose and glucosamine building blocks.

The advantage of route B over route A, however, is that there is greater flexibility in the protective group strategy (particularly with respect to the 2-amino functionality) and this leads to a more versatile scheme overall. A pattern of differential protection that is relatively easy to accomplish by constructing lactosamine from galactose and glucosamine may be more difficult to achieve on lactal or a 2-deoxy disaccharide derived from lactal. In such cases, the attractiveness of the pre-installed glycosidic bond is eroded by the extra manipulations required later on.

A survey of the protective groups on the building blocks used in route B reveals some common patterns (Table 2.2, entries 1-4, 6-8). The 2-OH of galactose is derivatized with an acyl group that will participate and enhance β-selectivity in glycosidation reactions. In most cases, the remaining hydroxyls of galactose are masked as benzyl ethers; these benzyl substituents serve as persistent protective groups that are orthogonal to the acyl protective group at the 2-OH, which must be selectively unmasked to permit α-fucosylation at that position.

Differential protection of the glucosamine building block is a more complex matter because it is the branch point of the tetrasaccharide determinant and its derivatization must facilitate several goals. The 6-OH, which requires persistent protection, is usually benzylated. Orthogonal protection is necessary for the 3-OH to allow selective unmasking for fucosylation at that position. The more commonly used groups for this purpose are the allyl ether, and the *p*-methoxybenzyl ether (subsequently found to be unstable during galactosylation[43, 64]); there are also examples in which Fmoc and Lev protective groups were employed. The unpopularity of acetyl protective groups in this case is likely due to the empirical finding that acetyl protective groups at the 3-OH of glucosamine seem to diminish the nucleophilicity of the 4-OH in glycosylations.[74]

The protective group for the 2-amino functionality should be selected with consideration of several factors: the influence on the nucleophilicity of the 4-OH[72] (for galactosylation); its activity as a neighbouring group participant for *β*-selective glycosidations at C-1 if such a reaction is desirable; and its steric bulk, which may affect the nucleophilicity of the 3-OH towards fucosylation (Figure 2.8).[65, 73] The syntheses reported earlier generally employed the acetamido group; the more recent syntheses use the trichloroethyl carbamate (NTroc) protective group.

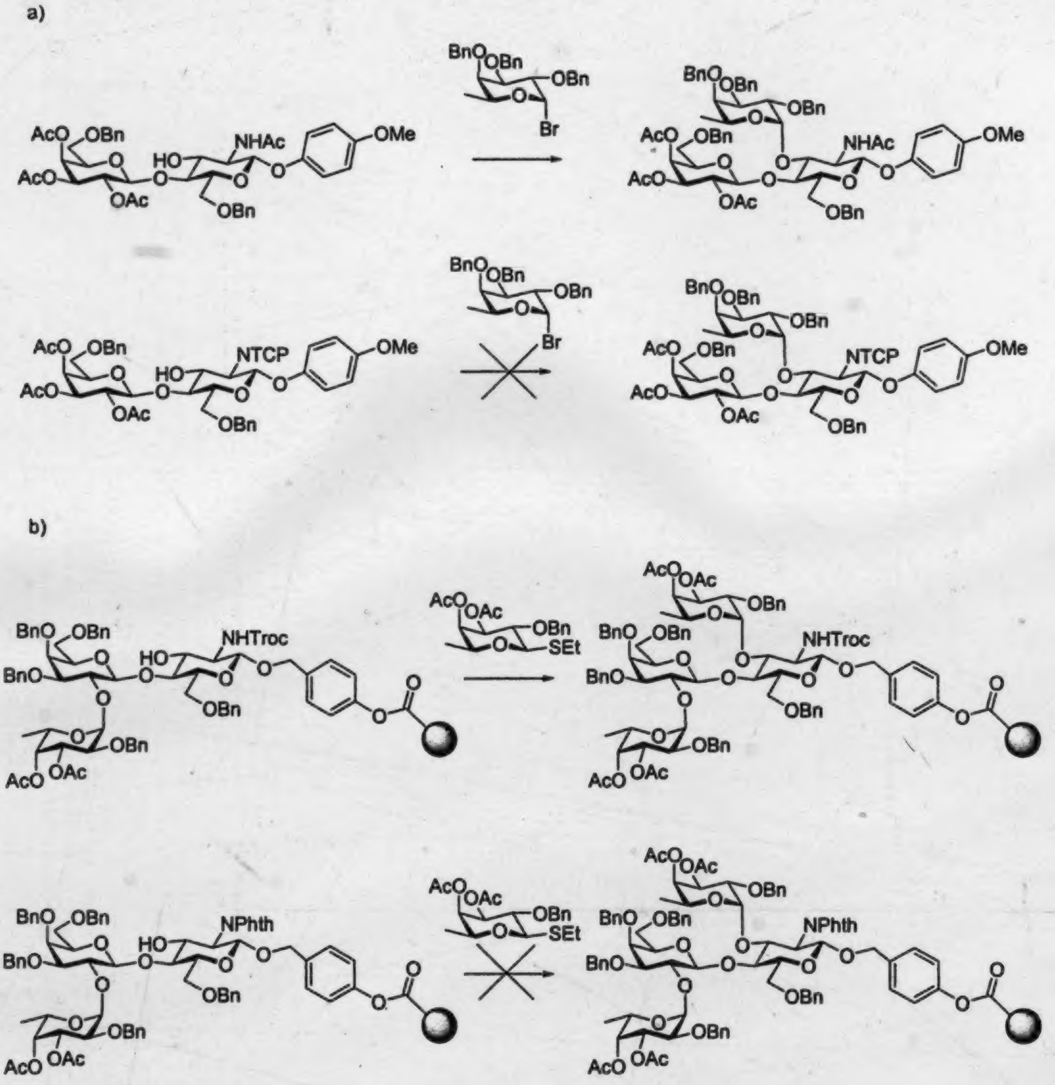

a)
AcO
OBn
AcO
OAc
HO
NHAc
OBn
OMe
BnO
OBn
OBn
Br
NTCP
b)
BnO
OBn
BnO
NHTroc
AcO
OAc
OBn
SEt
NPhth

Figure 2.8. *Examples of possible steric hindrance of fucosylation at the 3-OH by the* N-*phthaloyl or* N-*tetrachlorophthaloyl protective groups. (a) Fucosylation of a NHAc-protected lactosamine derivative is successful although attempted fucosylation of the analogous NTCP derivative is not.*[73] *(b) Fucosylation of a NHTroc-protected lactosamine derivative on polymeric support is successful although attempted fucosylation of the analogous NPhth derivative is not.*[65]

2.3. Retrosynthetic Strategy

We chose for several reasons to synthesize Le^y derivatives **1**, **2**, and **3** (Figure 2.1) as 2-(trimethylsilyl)ethyl (SE) glycosides. The SE functionality is comparable in size and hydrophobicity to the methyl group that is commonly employed as the aglycon in ligands for bioactivity investigations. However, in contrast to a methyl glycoside, a SE glycoside may be deblocked at the anomeric hydroxyl under relatively mild conditions to directly afford various types of donors[75] or the corresponding hemiacetal reducing sugar,[76] which in turn may be transformed into a donor (e.g. trichloroacetimidate).[77] Finally, the SE protective group is compatible with all the reactions in our synthetic strategy.

In the retrosynthetic analysis, we aimed to make optimal use of common building blocks for all three target compounds, as well as a common persistent protective group (benzyl ether) to simplify deblocking of the final compounds. The challenge in the synthesis of these target compounds is a function of their large size, the branched structure of Le^y, and the complexity of the building blocks.

Although compounds **1**, **2**, and **3** are essentially analogs of the Le^y determinant with varying aglycons, we chose not to design the Le^y tetrasaccharide as a common building block to be glycosidated with various acceptors. There is indeed precedence for such an approach (Figure 2.9), but the reports are not encouraging. The yield for glycosidation of the Le^y tetrasaccharide was in one case only 54%[65] and in the other, 69%.[58] Further, the isomeric Le^b thioglycoside was reported to be unstable and glycosidation of this tetrasaccharide donor was complicated by a competing elimination reaction in which the corresponding glucal was produced (Figure 2.9b).[78] We wished to avoid, as far as conceivable, reactions late in the synthesis that might prove problematic, and, therefore, projected instead the retrosynthetic analysis shown in Figure 2.10.

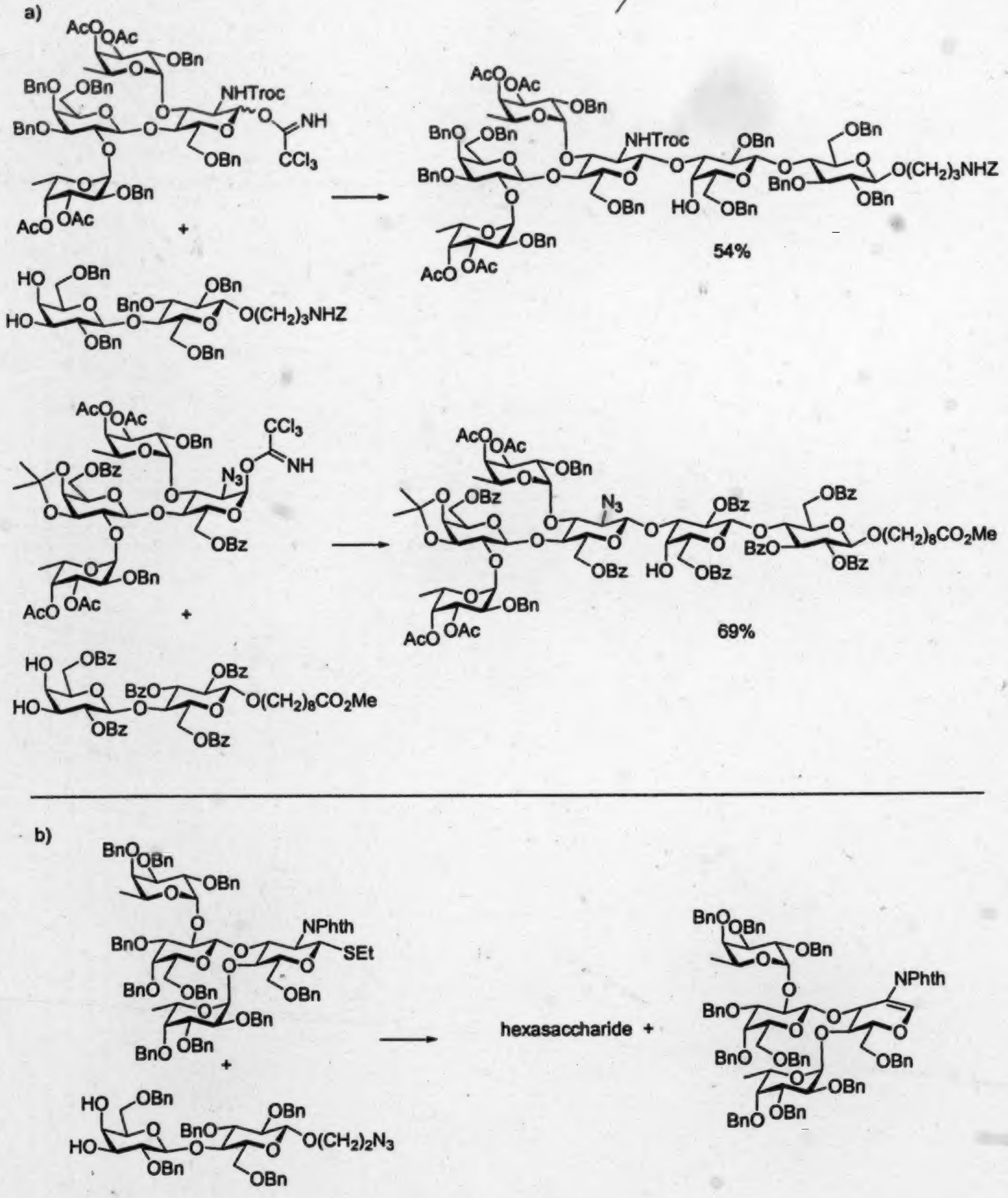

a)
NHTroc
CCl₃
54%
N₃
69%
b)
NPhth
SEt
hexasaccharide +
O(CH₂)₃NHZ
O(CH₂)₈CO₂Me
O(CH₂)₂N₃

Figure 2.9. *Examples of branched tetrasaccharide glycosidation reactions using (a) trichloroacetimidate Le^y donors and (b) a thioglycoside Le^b donor*

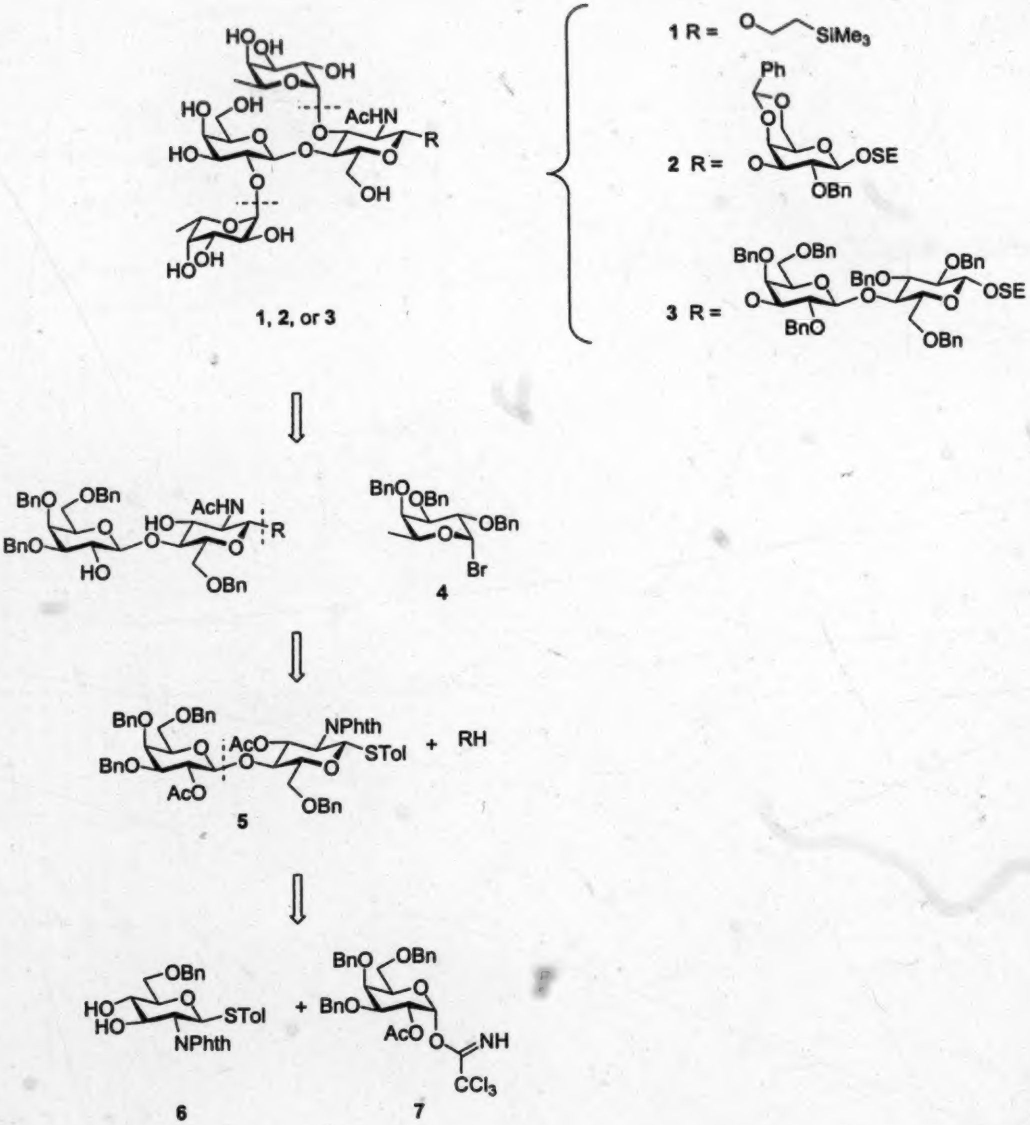
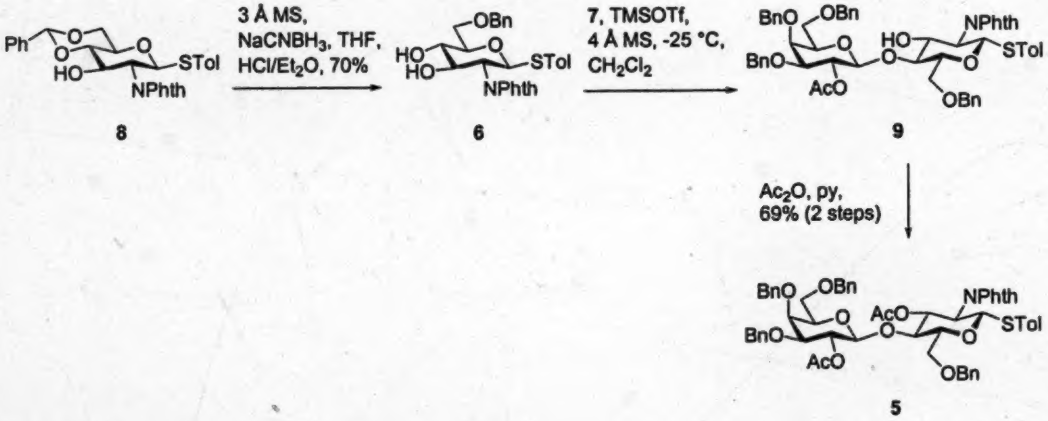

1, 2, or 3
1 R =
SiMe3
2 R =
Ph
OSE
OBn
3 R =
BnO
OBn
OSE
AcHN
R
4
Br
5
NPhth
STol
RH
6
7
CCl3
NH
AcO

Figure 2.10. *Retrosynthetic analysis of target compounds 1, 2 and 3*

The general retrosynthetic strategy (Figure 2.10) is similar to route B (Figure 2.7) discussed in the previous section. Initially, disconnection at both fucosidic bonds simultaneously yields a diol acceptor and fucosyl bromide **4**. The diol acceptor may be synthesized from lactosamine thioglycoside **5**, and acceptor RH. Lactosamine derivative **5** may be further disconnected into its monosaccharide building blocks: glucosamine derivative **6** and galactosyl donor **7**.

2.4. Results and Discussion

The key intermediate for all three Le^y targets is lactosamine derivative **5**, which we chose to synthesize from its monosaccharide components. Thus, thioglycoside **5** was obtained in 69% yield over 2 steps via chemo- and regioselective β-galactosylation at the 4-OH[73] of diol **6** with known trichloroacetimidate **7**,[79] followed by *O*-acetylation of the remaining free hydroxyl (Scheme 2.1). Donor **7** was synthesized in seven steps from D-galactose; diol **6** was prepared in 6 steps from D-glucosamine hydrochloride, the last step being the regioselective, reductive ring-opening of the benzylidene acetal[80] of known thioglycoside **8**.[81]


Ph
O
O
O
HO
STol
NPhth
8
3 Å MS,
NaCNBH$_3$, THF,
HCl/Et$_2$O, 70%
OBn
HO
HO
O
STol
NPhth
6
7, TMSOTf,
4 Å MS, -25 °C,
CH$_2$Cl$_2$
BnO
OBn
O
BnO
AcO
HO
O
NPhth
STol
O
OBn
9
Ac$_2$O, py,
69% (2 steps)
BnO
OBn
O
BnO
AcO
AcO
O
NPhth
STol
O
OBn
5

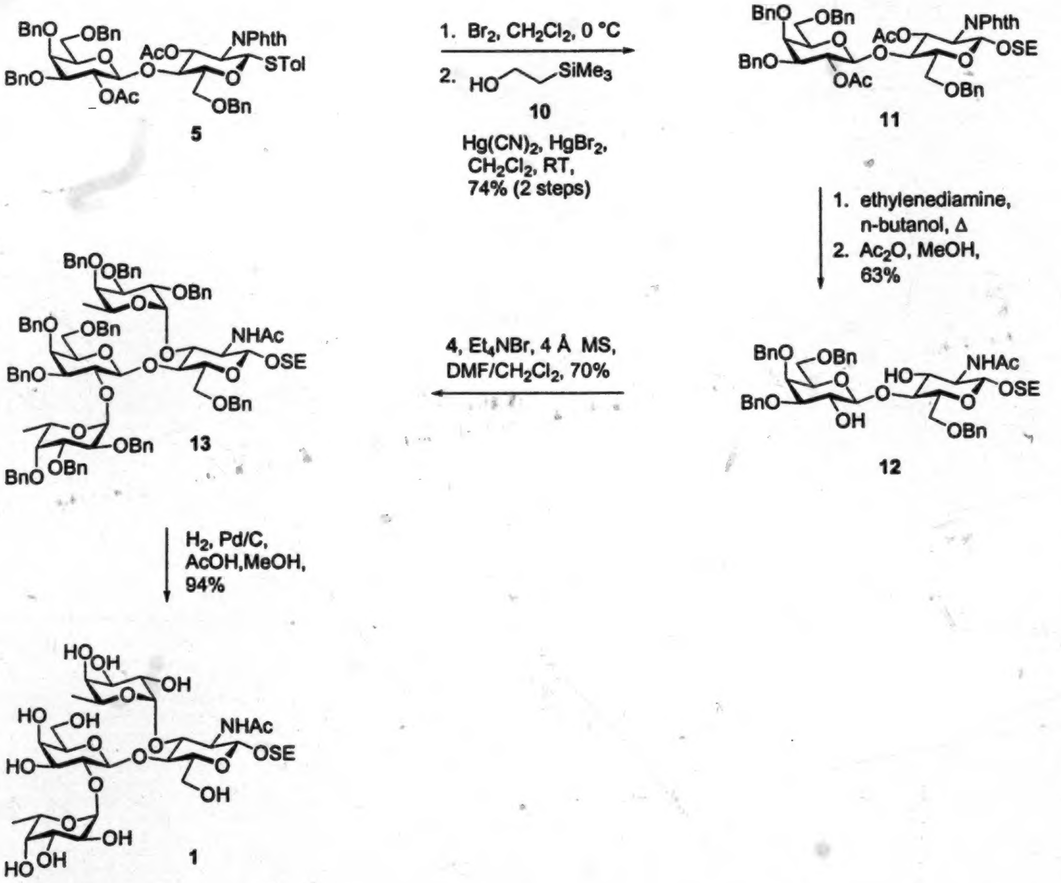
Scheme 2.1. *Synthesis of the lactosamine building block for Le^y derivatives*

Fucosyl bromide 4[82] is the other building block common to all three target compounds. Since 4 is relatively unstable and cannot be stored, it was prepared immediately before use by treatment of the corresponding thioglycoside with bromine. Ethyl 2,3,4-tri-*O*-benzyl-1-thio-*β*-L-fucopyranoside, the known thioglycoside analog of 4, was prepared in four steps from L-fucose.[83]

Synthesis of Le^y derivative **1** (Scheme 2.2) began with the treatment of lactosamine derivative **5** with bromine to generate the corresponding bromide donor which was subsequently *β*-glycosidated with 2-(trimethylsilyl)ethanol **10** under Helferich conditions[76] to afford **11** in 74% yield. Attempts to synthesize **11** by direct glycosidation of thioglycoside **5** with **10** in the presence of *N*-iodosuccinimide and either silver triflate or triflic acid were unsuccessful, although a similar aglycon transformation has been reported.[84]

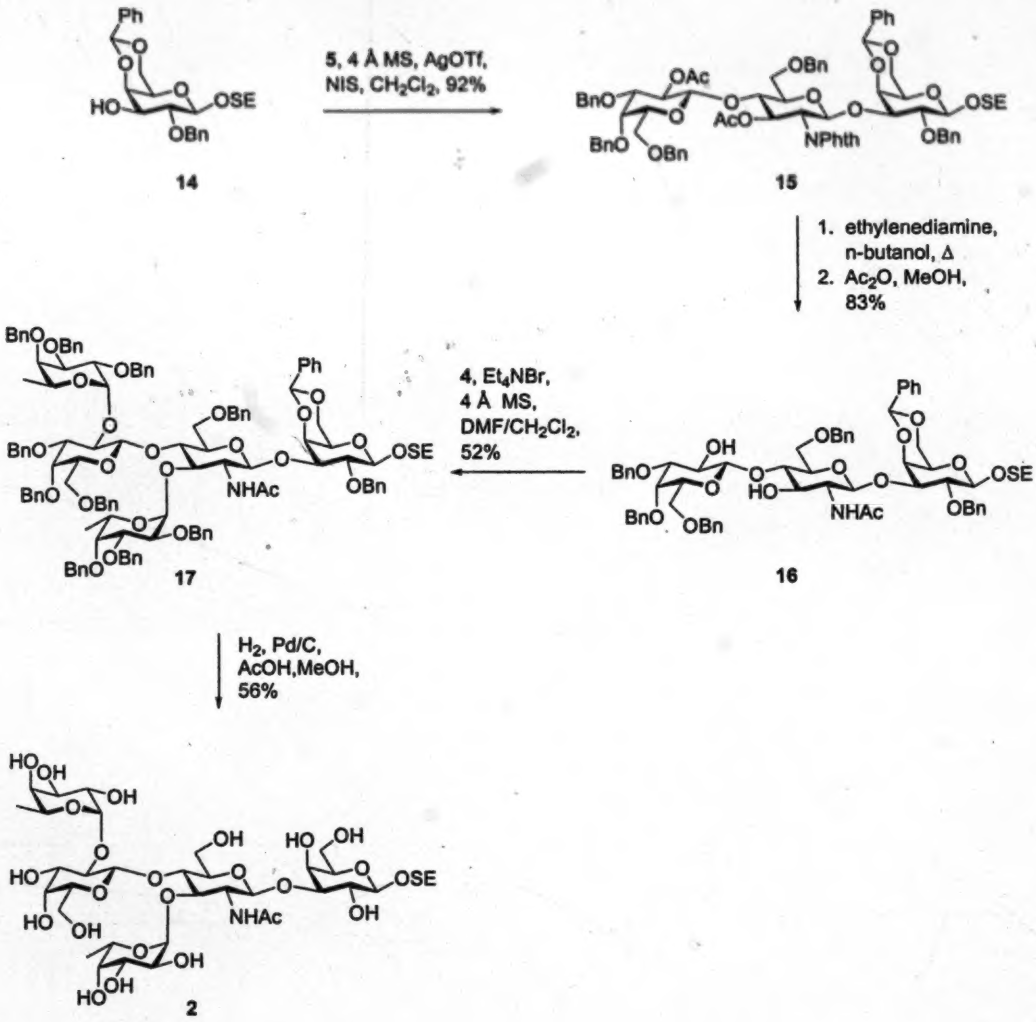
After sterically mediating the regioselectivity[73] of the condensation of **6** and **7**, and anchimerically assisting in the *β*-glycosidation reaction with **10**, the phthaloyl group masking the 2-amino functionality in **11** was no longer useful. Although fucosylation at the 3-OH in the presence of the 2-phthalimido functionality is not impossible,[43, 60] there have been reports of unsuccessful attempts (Figure 2.8).[65, 73] In light of the conflicting reports, we decided not to risk steric complications and thus treated **11** with ethylenediamine to simultaneously cleave the phthaloyl group as well as the acyl protective groups at the 3-OH and 2'-OH. The resulting 2-amino intermediate was immediately and selectively *N*-acetylated with acetic anhydride in methanol to afford diol **12** in 63% yield over the two steps.

Di-*α*-fucosylation of diol **12** with freshly-prepared fucosyl bromide **4** was effected under halide catalysis conditions[85] to give the protected Le^y derivative **13** in 70% yield, which is comparable to the yield (60% - 86%) reported by others for difucosylation of similar 2-acetamido lactosamine acceptors[43, 53, 61, 62, 64] and considerably better than the yield reported for difucosylation of lactal (51%).[56, 57] Target compound **1** was obtained from tetrasaccharide **13** by catalytic hydrogenolysis of the benzyl ether protective groups using Pd-C in acetic acid-methanol.


5
1. Br_2, CH_2Cl_2, 0 °C
2. HO~SiMe$_3$ 10
$Hg(CN)_2$, $HgBr_2$, CH_2Cl_2, RT, 74% (2 steps)
11
1. ethylenediamine, n-butanol, Δ
2. Ac_2O, MeOH, 63%
12
4, Et_4NBr, 4 Å MS, DMF/CH_2Cl_2, 70%
13
H_2, Pd/C, AcOH,MeOH, 94%
1
BnO
OBn
OAc
AcO
NPhth
STol
OSE
NHAc
HO
OH

Scheme 2.2. *Synthesis of Le^y tetrasaccharide 1*

Synthesis of the pentasaccharide Le^y derivative **2** is similar to that of **1** (Scheme 2.3). In this case, however, we were able to directly glycosidate lactosamine **5** without first converting it into a bromide donor. Thus, NIS-promoted glycosylation of known galactose derivative **14**[76] (synthesized in eight steps from D-galactose) with thioglycoside **5** proceeded smoothly to furnish trisaccharide **15**.


14
5, 4 Å MS, AgOTf, NIS, CH2Cl2, 92%
15
1. ethylenediamine, n-butanol, Δ
2. Ac2O, MeOH, 83%
16
4, Et4NBr, 4 Å MS, DMF/CH2Cl2, 52%
17
H2, Pd/C, AcOH,MeOH, 56%
2

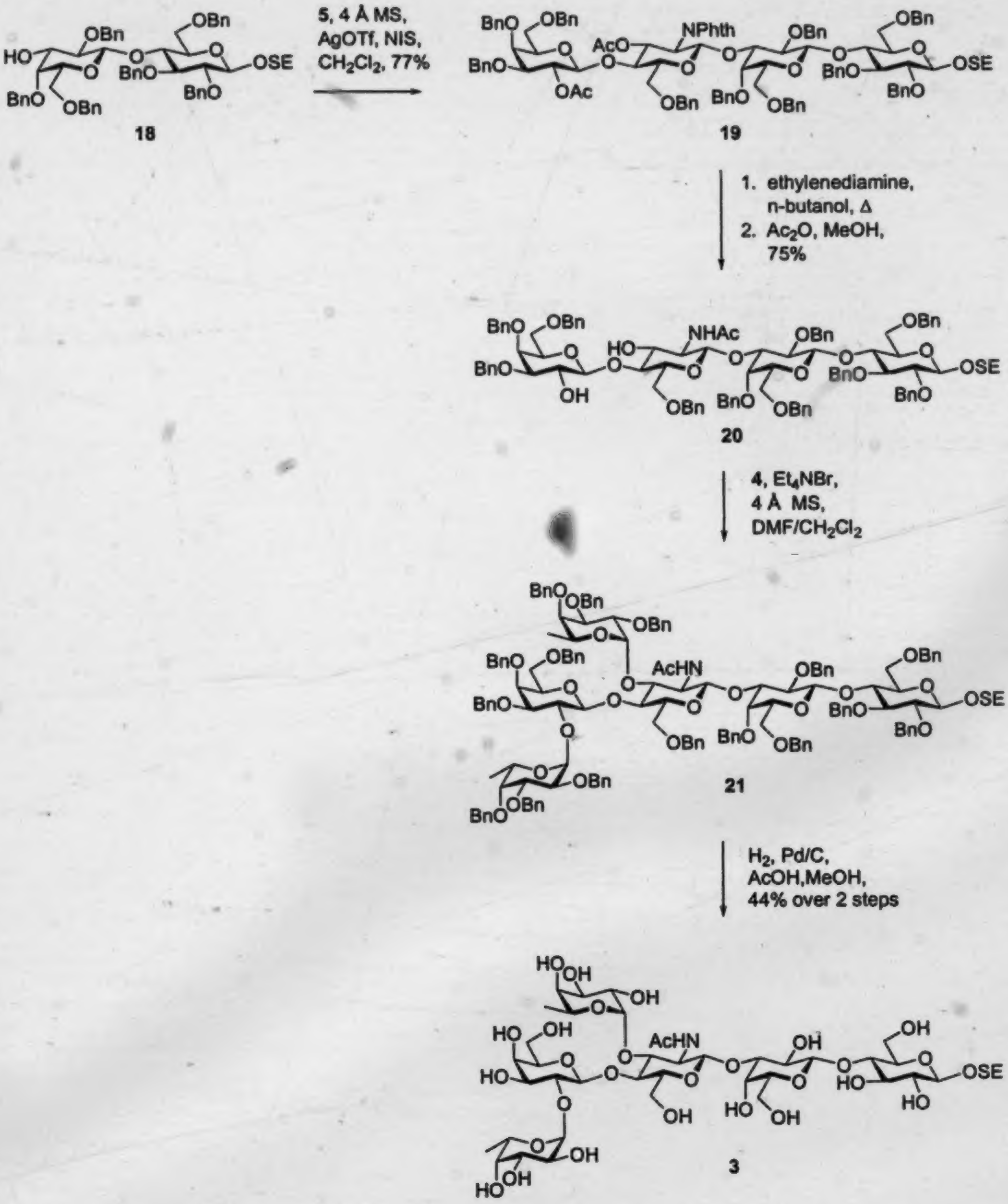
Scheme 2.3. *Synthesis of Le^y pentasaccharide 2*

Heating to reflux a butanolic solution of trisaccharide **15** in the presence of excess ethylenediamine and subsequent *N*-acetylation with acetic anhydride in methanol effected de-*O*-acetylation as well as transformation of the 2-phthalimido into the 2-acetamido group to give diol **16** in good yield. Halide-ion catalyzed di-α-fucosylation of diol **16** with fucosyl bromide **4** afforded the desired pentasaccharide **17**, in a modest yield (52%) equivalent to 72% efficiency for the installation of each fucosidic linkage.

The reason for the significantly lower yield obtained for the difucosylation of trisaccharide **16** to give pentasaccharide **17**, compared to the yield for the similar conversion of disaccharide **12** to tetrasaccharide **13**, is not clear. Some groups that have synthesized Le^y analogs[53, 54, 60] have identified the H-type 2 trisaccharide as the major side product generated during similar difucosylation reactions; Depré et al. speculated that observation of this trisaccharide (α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc) may indicate inhibition of fucosylation at the 3-OH by fucosylation at the 2'-OH.[60] It is possible that underfucosylation was a greater problem for trisaccharide **16** than for disaccharide **12** and contributed to a lower yield of **17** compared to **13**; however, we did not try to isolate a side product that would confirm this.

Catalytic hydrogenolysis of **17** over Pd-C in acetic acid-methanol cleaved all eleven benzyl ethers as well as the 4',6'-*O*-benzylidene acetal to afford Le^y pentasaccharide **2** in only 56% yield. The low yield of the global *O*-deprotection step is attributed to loss during chromatographic purification, because subsequent experiments in which pentasaccharide **17** was globally deblocked and directly *O*-acetylated (without purification of intermediate **2**) gave a yield of 87% over the two steps.

The synthesis of hexasaccharide Le^y derivative **3** is analogous to the syntheses of **1** and **2** and employs the same key building blocks (Scheme 2.4). Activation of thioglycoside **5** with NIS-silver triflate in the glycosylation of known alcohol **18** (prepared from lactose in eight steps[86, 87]) rendered tetrasaccharide **19** in 77% yield. Manipulation of the protective groups at N-2'', O-3'', and O-2''' in two steps (treatment with ethylenediamine followed by acetic anhydride in methanol) converted **19** into diol **20** in 75% yield. Addition of an excess of fucosyl donor **4** to acceptor **20** under halide ion catalyzed glycosidation conditions gave protected Le^y hexasaccharide **21**, which proved difficult to purify by chromatography despite repeated attempts. Palladium-catalyzed reductive cleavage of the sixteen benzyl ether protective groups of **21** furnished **3**, which was purified by reverse-phase HPLC. The yield of **3** over both the difucosylation and global deprotection reactions was 44%.


18
5, 4 Å MS,
AgOTf, NIS,
CH_2Cl_2, 77%
19
1. ethylenediamine,
n-butanol, Δ
2. Ac_2O, MeOH,
75%
20
4, Et_4NBr,
4 Å MS,
DMF/CH_2Cl_2
21
H_2, Pd/C,
AcOH,MeOH,
44% over 2 steps
3

Scheme 2.4. *Synthesis of Le^y hexasaccharide 3*

The overall yields obtained for Le^y analogs **1**, **2** and **3** (Schemes 2.2, 2.3, and 2.4) using key building blocks **4** and **5** were 31%, 22%, and 21%, respectively. These yields are comparable to those reported by other groups who synthesized similar Le^y analogs. Polymer-supported synthesis afforded a protected Le^y tetrasaccharide in 30-35% yield, while subsequent manipulations leading to the final solution-phase glycosylation produced a protected hexasaccharide target compound in 38% yield.[65] Reactivity-based one-pot synthesis of a similar target furnished a protected Le^y hexasaccharide efficiently (44% yield) while the subsequent three-step deprotection sequence was effected in only 25% yield.[66]

COSY, TOCSY, and 2D TROESY NMR data were required for assignment of the signals observed in the 1D ^1H NMR spectrum of **1**. Observation of a cross peak in the 2D TROESY spectrum correlating the 2-*N*-acetyl protons with H-1' of the O-3 fucosyl residue permitted differentiation between the two fucose residues of the Le^y determinant. The spectrum for **1** was in agreement with reported ^1H NMR data for a tetrasaccharide Le^y derivative possessing a different aglycon.[43] Because the chemical shifts for analogous protons in **1**, **2**, and **3** were relatively similar, comparison of the ^1H NMR spectra of the three analogs facilitated the assignment of the signals arising from the two galactose residues and two fucose residues found in the extended Le^y compounds **2** and **3**.

Interpretation of the ^1H NMR spectra for the protected Le^y analogs **13**, **17** and **21** was carried out in a similar manner. Discrimination between the fucose moieties in **13** was possible with 2D TROESY experimental data; assignment of the ^1H NMR spectra of protected analogs **17** and **21** was based on the assignments made for **13**.

Chapter 3

FAC/MS Screening of Le^y Analogs as Ligands for Antibody B3

3.1. Background

3.1.a. Binding Properties of Antibody B3

The selective tumor reactivity exhibited by mouse monoclonal antibody B3[29] has generated interest in potential applications of B3 to cancer diagnosis[17] and therapy.[30] However, the origin of its higher tumor specificity compared to other Le^y-directed antibodies is not understood. The natural antigen for antibody B3 has not been isolated, nor has the binding site of B3 been investigated.

Reported studies on the binding properties of antibody B3 have been only qualitative.[29] First, human tissue selectivity was evaluated via immunohistochemical experiments; the results indicate that B3 reacts more intensely with many carcinomas than to normal human tissues. Next, the expression of the natural antigen on cancer cells was characterized in two ways: immunofluorescence analysis of living cells and immunoprecipitation. From these two experiments, it was determined that the natural antigen recognized by B3 is expressed homogeneously on the exterior cell surface and that the epitope may be carried by diverse glycoproteins (ranging in molecular weight below 40,000 and above 200,000). Finally, the chemical structure of the carbohydrate epitope was deduced by ELISA on a panel of synthetic glycoconjugates. Comparison of bound structures (i.e., Le^y, Le^a-Le^x, di-Le^x, and tri-Le^x)[*] with unrecognized structures similar to the Le^y determinant (i.e., Le^x, H Type 2, Le^b)[*] led to the proposal that the fucose moiety α(1→3) linked to GlcNAc is necessary, but not sufficient, for B3 recognition. It appears that either substitution at the 3-OH of galactose or (even better) the presentation of a second fucose moiety at the 2-OH of galactose, is also required.[29]

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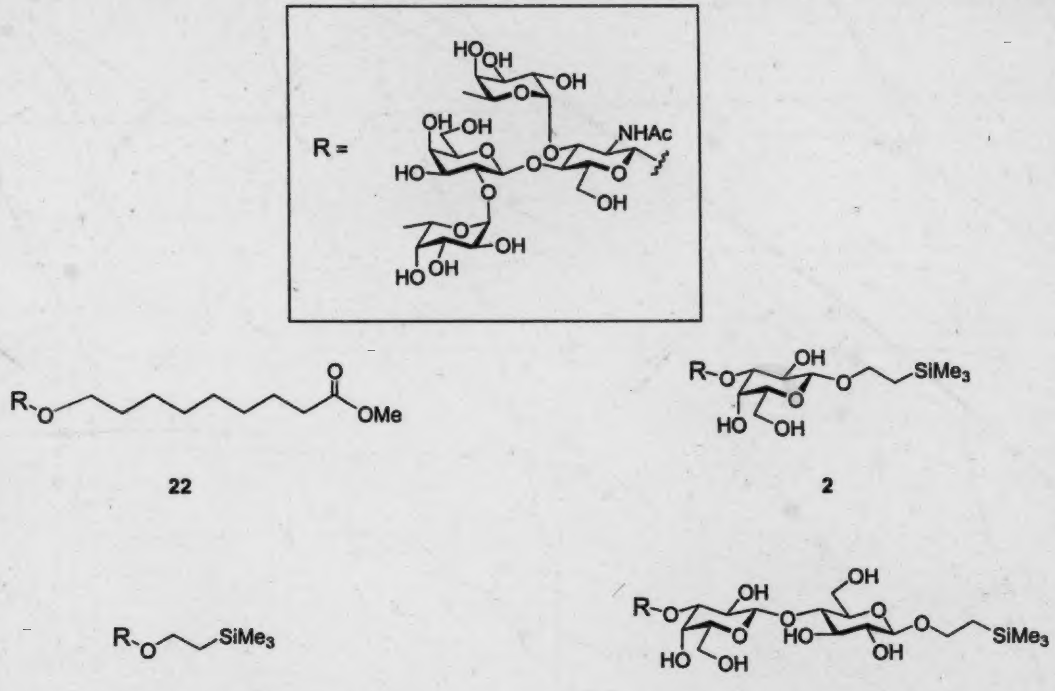
[*] Refer to Figure 1.2 for the structures of the blood-group and related antigens.

We would like to explore the possibility that B3 owes its tumor specificity to the recognition of additional sugars at the reducing end of the Le^y tetrasaccharide determinant, thereby exploiting some difference in context, presentation, or accessibility between the expression of the same determinant on normal cells and cancer cells.

This hypothesis--that the molecular basis for the tumor specificity exhibited by some anti-Le^y monoclonal antibodies is a binding site capable of extended recognition of the Le^y structure expressed on cancer cells--has been proposed before for B3 and related antibodies. Using a combination of computer modeling and relevant crystal structure data, Blaszczyk-Thurin *et al.* identified the shared structural features among the binding sites of a few representative anti-Le^y antibodies and concluded that the GlcNAc residue and additional hexoses at the reducing end of the Le^y tetrasaccharide might be important for specificity.[32]

The studies performed by Blaszczyk-Thurin *et al.* were theoretical in nature. The most direct observations supporting extended structure recognition arise from data on mouse monoclonal antibody BR96. BR96 was raised against human breast cancer cells and is selectively directed to Le^y-expressing cancers.[23] The X-ray crystal structure of the antigen-binding fragment (Fab) of BR96 in a complex with its synthetic antigen, 8-methoxycarbonyloctyl Le^y derivative **22** (Figure 3.1), shows that the alkyl aglycon interacts extensively with the antibody (Figure 3.2). It was inferred that in a complex of BR96 with the natural antigen, a carbohydrate extension at the reducing end of Le^y would fill the structural role of the synthetic alkyl aglycon.[33]



22 2 1 3

Figure 3.1. *Analogs of the Le^y determinant (R) for which B3 binding affinities were estimated by FAC/MS*

When evaluating the potential importance of extended structure recognition, two points are noteworthy. One is that while there are antibodies directed to larger carbohydrate determinants of which the Le^y motif is a part (Figure 3.3), these antibodies bind a different epitope than the one recognized by BR96, B3, and related anti-Le^y antibodies. For BR96 and related antibodies, the minimal Le^y structure is both necessary and sufficient for binding. It may be possible, however, that antibody recognition of carbohydrate residues extending beyond Le^y enhances only slightly the energetics of the interaction, but has a significant influence *in vivo* when multivalent interactions with the tumor cell surface or when interactions of other proteins (e.g., of the complement cascade) with the antigen-bound antibody may be factors. The second important point is that in the natural antigen, the tetrasaccharide Le^y is always a substructure of a macromolecule. The Le^y motif is a peripheral group carried on an oligosaccharide chain that is conjugated to protein or lipid (Figure 3.4); consequently additional structures at the reducing terminus are always present in the natural antigen. Unfortunately, since the core oligosaccharide carrier is usually heterogeneous in sequence, it is possible only to generalize about the identity of the hexoses adjacent to Le^y in the natural antigen that may be recognized by tumor-specific antibodies such as B3.

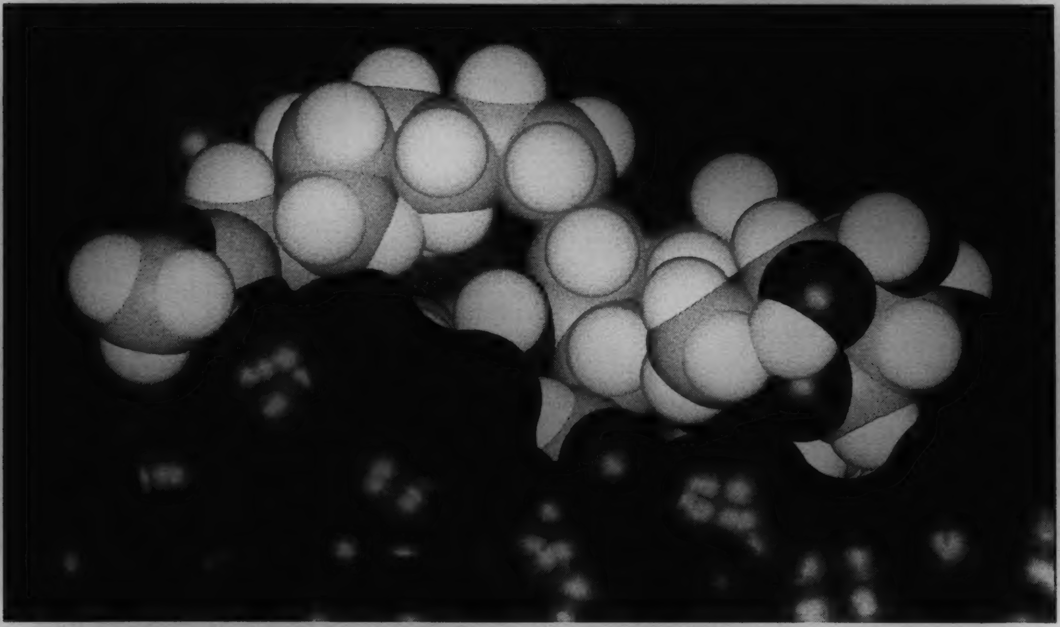


Figure 3.2. *Model based on the X-ray structure of the binding site of chimeric BR96 complexed with 8-methoxycarbonyloctyl Le^y derivative 22.*[33] *The Connolly surface of the antibody is displayed in blue; the CPK model of the Le^y antigen is displayed in green (carbon), red (oxygen), and white (hydrogen). The aglycon of Le^y derivative 22 contributes favourably to the interaction with BR96.*

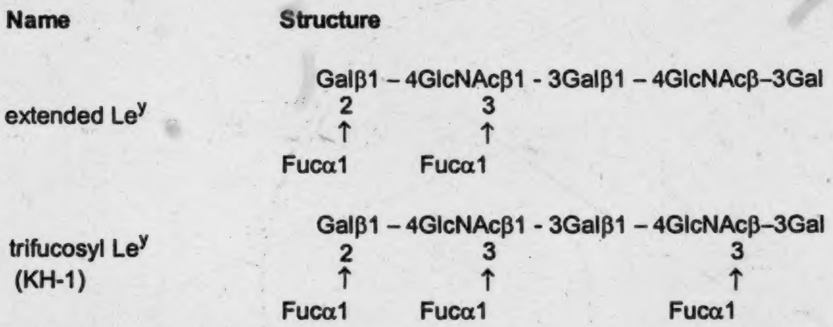

Name
Structure
extended Le^y
Galβ1 – 4GlcNAcβ1 - 3Galβ1 – 4GlcNAcβ–3Gal
2
3
Fucα1
Fucα1
trifucosyl Le^y
(KH-1)
Galβ1 – 4GlcNAcβ1 - 3Galβ1 – 4GlcNAcβ–3Gal
2
3
3
Fucα1
Fucα1
Fucα1

Figure 3.3. *Antigenic determinants incorporating the Le^y motif. Trifucosyl Le^y, though sharing structural similarities with Le^y, falls into a different class of antigens. Unlike the Le^y antigen that is detected in a number of normal tissues but is very highly expressed in some tumor cells, trifucosyl Le^y is highly expressed in tumor cells but absent in progenitor cells.*[4]

We synthesized compounds **1**, **2**, and **3** to probe the influence of one or two additional hexoses at the reducing terminus of Le^y: either galactose alone or the disaccharide lactose (Galβ(1→4)Glcβ). The quantitative evaluation of the binding affinity of monoclonal antibody B3 for four synthetic Le^y analogs (Figure 3.1) was achieved via frontal affinity chromatography coupled online to electrospray mass spectrometry (FAC/MS).[88] Our studies test the proposal of larger epitope recognition[32] and extend the earlier, qualitative investigations concerning the epitope of antibody B3.[21, 29]

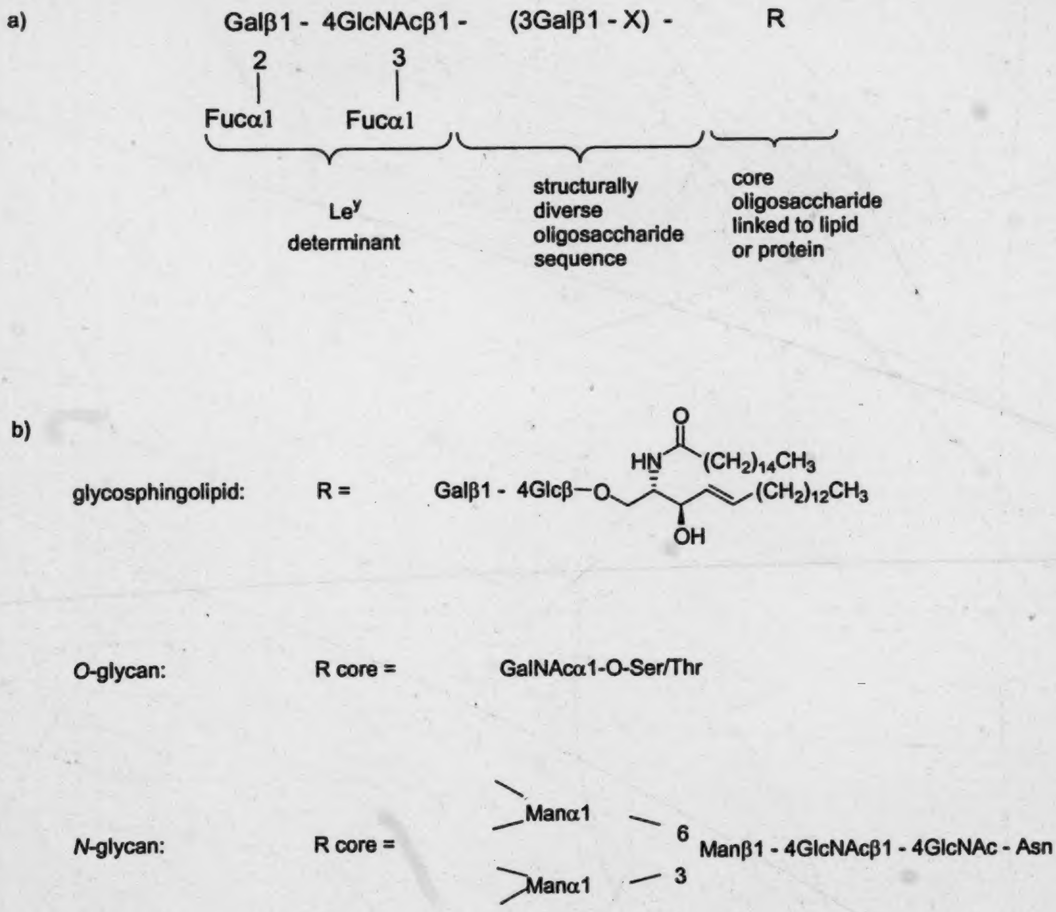

a)
Galβ1 - 4GlcNAcβ1 - (3Galβ1 - X) - R
2
3
Fucα1
Fucα1
Le^y determinant
structurally diverse oligosaccharide sequence
core oligosaccharide linked to lipid or protein
b)
glycosphingolipid:
R =
Galβ1 - 4Glcβ—O
HN
O
(CH2)14CH3
(CH2)12CH3
OH
O-glycan:
R core =
GalNAcα1-O-Ser/Thr
N-glycan:
R core =
Manα1
6
Manα1
3
Manβ1 - 4GlcNAcβ1 - 4GlcNAc - Asn

Figure 3.4. *Generalized structure of the natural* Le^y *antigen. (a) The* Le^y *tetrasaccharide is a peripheral structure that is conjugated to either protein or lipid, sometimes via an oligosaccharide spacer of variable length. The spacer is heterogeneous in sequence. It begins with galactose, and frequently consists of polylactosamine, i.e. repeating units of [3Galβ(1→ 4)GlcNAcβ(1→ 3)]. The reducing end of the oligosaccharide sequence depends on the nature of the* Le^y *glycoconjugate: the general core structures for glycosphingolipids, and glycoproteins are shown in (b).*

3.1.b. FAC Coupled Online with Mass Spectrometry

Micro-scale FAC/MS is a screening method that was recently developed for the assay of compound libraries.[88] We chose to use this method to investigate the recognition of synthetic Le^y analogs by mAb B3 because it permits rapid and quantitative estimates of dissociation constants while minimizing the amount of material consumed.

Rapid evaluation of binding activity is possible with FAC/MS because a mixture of compounds can be characterized simultaneously in a single run, and the duration of a typical run using a miniaturized affinity column is a few minutes. A potential disadvantage to FAC/MS is that the ligand-receptor interaction must occur at a solution-solid interface, and this interaction does not model real recognition events unless the natural protein receptor is membrane-bound. However, the interfacial nature of the interaction seems not to interfere significantly with the accuracy of the dissociation constants (K_ds) measured by this method. It has been found that the constants obtained via FAC are consistent with values obtained from solution methods, such as equilibrium constants obtained via equilibrium dialysis,[89] dissociation constants determined by microcalorimetry,[88] and inhibition constants (K_Is) determined in solution via radiochemical assay. In the latter case, the relative ranking of compounds was identical although the K_ds were found to be 2-10 times lower than the K_Is.[90]

In FAC/MS, a solution containing a mixture of potential ligands at a constant, micromolar concentration is applied continuously to a micro-scale column on which a protein receptor is immobilized. The column is directly coupled to an electrospray mass spectrometer that is programmed to monitor two parameters in the effluent from the column: the intensity and the mass-to-charge ratio (*m/z*) of the ligands. The compounds break through the column in an order reflecting their relative binding activities: the weakest binders will break through earlier while the strongest binders will be retained to a greater extent and, therefore, exit the column later (Figure 3.5).

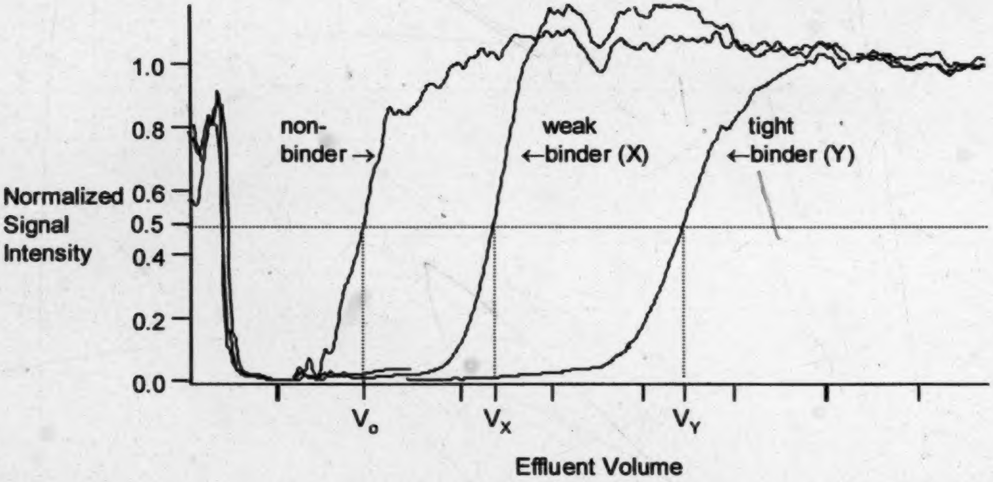


Figure 3.5. *Example of a FAC/MS chromatogram obtained from the continuous infusion of a mixture of 3 ligands of various activities through an affinity column. More active ligands are retarded on the column to a greater extent and consequently break-through later. One non-binding compound should be included in every analyte mixture to serve as a marker of the break-through volume in the absence of specific interaction (V_o) for the accurate measurement of retention volumes of active ligands ($V-V_o$). Selected ion monitoring of the effluent from the column by electrospray MS allows the extraction of frontal chromatograms for each* m/z *of interest, shown here overlaid and normalized.*

The thermodynamic theory relating frontal chromatographic data to dissociation constant estimation was developed by Kasai *et al.*[89] Equation 1 applies when the column is in a state of dynamic equilibrium (which corresponds to the plateau in the frontal chromatogram); this situation is treated as a simple equilibrium between the free binding partners (ligand A and immobilized receptor B) and the bound complex (A-B). $[A]_o$ is the initial concentration of A being applied to the column, $[B]_o$ is the concentration of immobilized receptor (assumed to be distributed uniformly over the column), B_t is the total amount of immobilized receptor capable of binding A, $(V-V_o)$ is the retention volume of A due to specific interaction with B, and v is the bed volume of the column.

Equation 1 can be rearranged into a more useful form, Equation 2.

Equation 1:

$$K_d = \frac{[A][B]}{[AB]} = \frac{[A_o]\{[B_o] - [A_o](V - V_o)/v\}}{[A_o](V - V_o)/v} = \frac{B_t}{V\text{-}V_o} - [A]_o$$

Equation 2:

$$\frac{1}{[A]_o\,(V\text{-}V_o)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_o} + \frac{1}{B_t}$$

In practice, if both K_d (a property of ligand A) and B_t (a characteristic of the affinity column) are not known, they may be determined simultaneously from a set of experiments in which the retention volume of ligand A is measured for various concentrations of A (Figure 3.6a) infused through the FAC column. Generation of a plot (Figure 3.6b) using the experimental data gives a line which has a y-intercept equal to the reciprocal of B_t and a slope whose value gives K_d/B_t. The linear relationship between the reciprocal of $[A]_o(V\text{-}V_o)$, i.e. adsorbed A, and the reciprocal of $[A]_o$ is described by Equation 2.

Once the affinity column is characterized (i.e. the specific binding capacity B_t is known), the rapid, quantitative evaluation of a mixture is possible if the initial concentration of each compound in the mixture is known. Thus, if a mixture is applied to a FAC/MS column, determination of the retention volumes for each analyte in the mixture allows the estimation of K_d^{mix} for each compound in the presence of the others. Theoretically, if each analyte in the mixture is infused through the column at a concentration much lower than its K_d, then K_d^{mix} is a good estimate of K_d since in such cases, one may consider that each analyte is subjected to chromatography using, in effect, separate columns.[89]

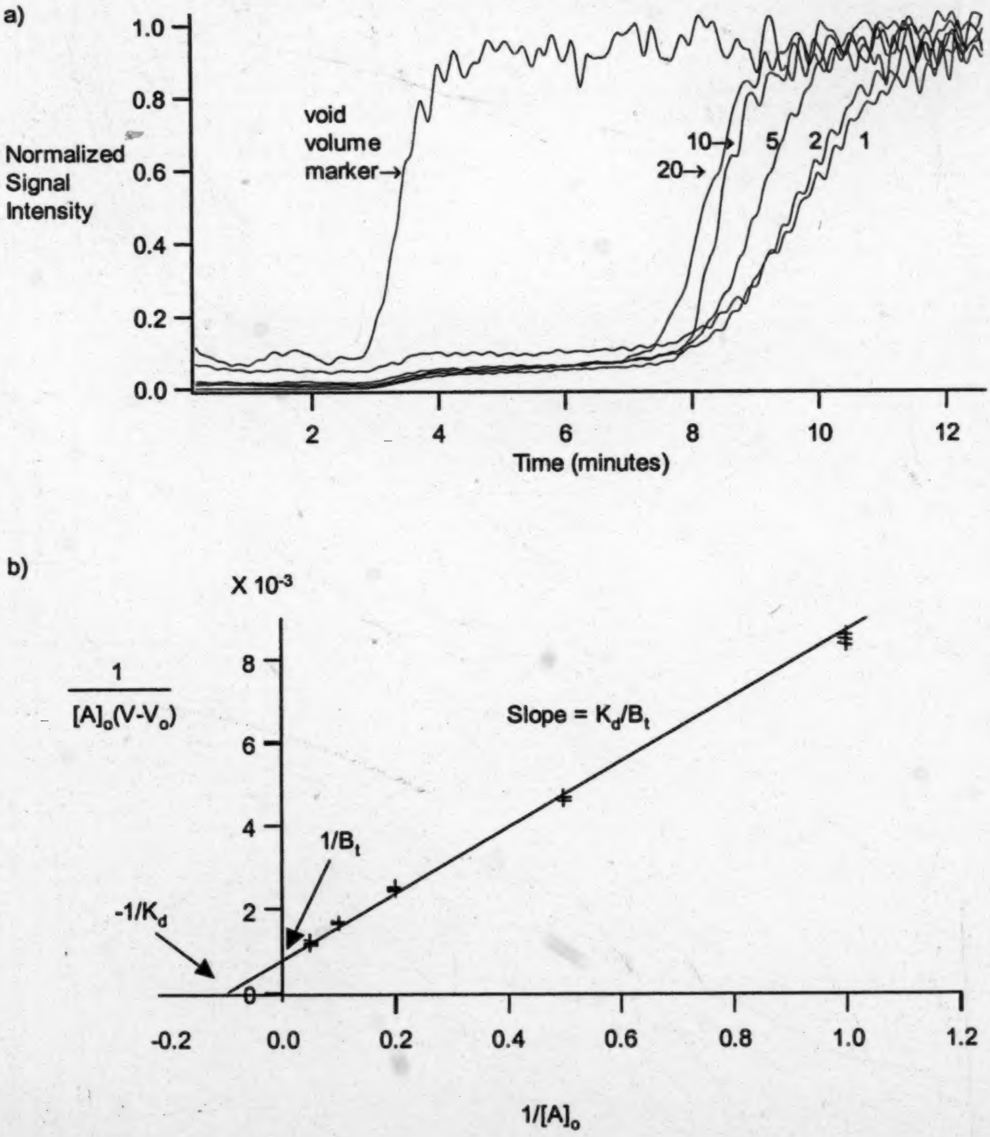


Figure 3.6. *a) Overlay of frontal profiles obtained following the infusion of 5 different concentrations of ligand A ($[A]_o$ =1, 2, 5, 10, or 20 μM) through an affinity column. Measurement of the retention volume ($V-V_o$) for each concentration of the analyte $[A]_o$ allows the determination of K_d and B_t as shown in (b). b) Plot illustrating the linear relationship between the reciprocal of adsorbed A and the reciprocal of $[A]_o$ as described by Equation 2. K_d is obtained from the slope and B_t is obtained from the y-intercept of the line.*

Although the shape of the frontal profile was not considered in the derivation of Equation 1, and therefore does not contribute to the quantitative analysis of binding interactions in FAC, it can provide valuable, qualitative information. For example, an unexpectedly diffuse (gently sloping) front is an indication of the presence of a mixture of unresolved, isomeric species with similar affinity for the immobilized receptor.[91] Also, the observation of a "bump" in the frontal profile corresponding to an increase in the analyte concentration beyond its infusion concentration, instead of the usual plateau (e.g. the profile for weak binder X in Figure 3.5), is an indication that the ligand whose profile is thus affected was displaced by a stronger, competitive ligand in the analyte mixture.[90]

3.2. Assay of Lewis Y Analogs by FAC/MS

Preparation of the microscale affinity column began with the biotinylation of antibody B3 (generously donated by I. Pastan at NIH). In order to protect the binding site of the protein from chemical modification, a solution of antibody B3 and excess Le^y derivative **22**[*] was equilibrated before the addition of biotinylating agent. Estimation of the efficiency of the reaction was *via* MALDI mass spectrometric analysis; the difference in mass between biotinylated B3 and native B3 corresponded to the incorporation of eleven biotin moieties per antibody.

Biotinylated B3 was immobilized (via the strong, non-covalent reaction of biotin with the streptavidin) on a microscale column packed with controlled-pore glass beads conjugated to streptavidin (CPG-SA beads). Unreacted streptavidin sites in the B3 affinity column were blocked with D-biotin, which was also used to saturate all sites in a similar-sized, blank column.

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[*] gift from Dr. O. Hindsgaul

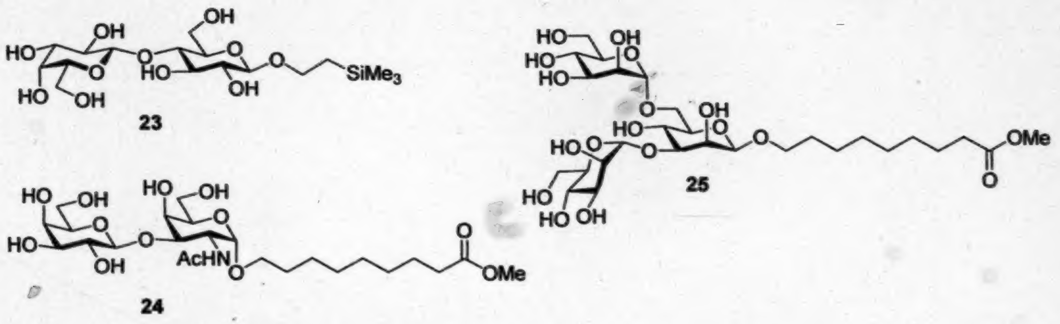

Figure 3.7. *Void markers for the B3 affinity column. These compounds were used to ascertain the elution volume in the absence of specific binding.*

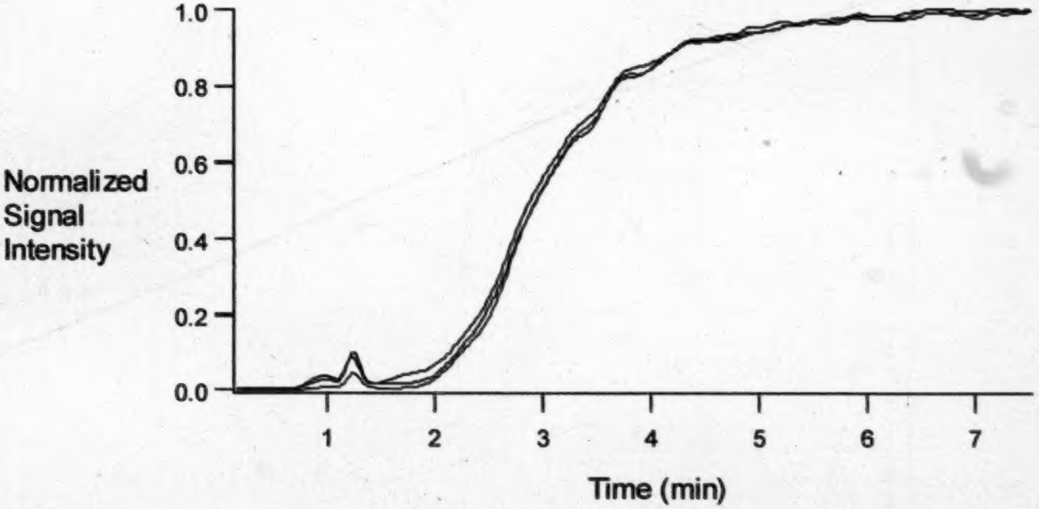

Figure 3.8. *Void volume marker FAC/MS assay. The data shown here was drawn using the IGOR program. Normalized, extracted ion chromatograms, obtained by monitoring the effluent from the infusion (8 μL/min) of a mixture of compounds* ***23*** *(2 μM, red),* ***24*** *(1.38 μM, blue), and* ***25*** *(1.38 μM, black) in buffer (10 mM NH_4OAc, 1 mM NaCl, pH 7.4) through a B3 affinity column, are shown overlaid. The three compounds, none of which are expected to show specific binding to B3, break through the affinity column at the same time. Lactose derivative* ***23*** *was deemed to be a suitable "void" reference against which specific retention could be measured in subsequent assays.*

Two control FAC/MS experiments were performed prior to the assay of the synthetic Le^y analogs **1**, **2**, **3**, and **22** (Figure 3.1) as ligands for antibody B3. First, the suitability of disaccharide **23**[76] as a marker of the break-through volume in the absence of specific binding to B3 was confirmed. Known lactose derivative **23** (synthesized from lactose in four steps as described previously[76]) was infused through the B3 affinity column in a mixture with two other sugars that bear no resemblance to the Le^y determinant and consequently were not expected to bind to antibody B3: 8-methoxycarbonyloctyl *β*-D-galactopyranosyl-(1→ 3)-2-acetamido-2-deoxy-*α*-D-galactopyranoside (**24**)[•] and 8-methoxycarbonyloctyl *α*-D-mannopyranosyl-(1→ 3)-[*α*-D-mannopyranosyl-(1→ 6)]-*β*-D-mannopyranoside (**25**)[•]. The three compounds were retained to the same extent, confirming the void volume of the column (Figures 3.7, 3.8).

In the second control experiment, a mixture of Le^y analogs (**2**, **3**, and **22**) and the void volume marker **23** were infused through a blank column of immobilized D-biotin. All compounds broke through the control column simultaneously, indicating that in the absence of immobilized antibody B3, the Le^y analogs and the void marker are retained to the same extent by non-specific interactions (if any) with the column matrix (Figure 3.9).

Having established that specific binding in FAC/MS assays using the B3 affinity column could be positively identified, we proceeded to characterize the affinity column by determining its binding capacity. Infusion of compound **2** at varying concentrations (1, 2, 5, 10 and 20 μM) through the B3 affinity column and triplicate measurement of the corresponding break-through volumes (with respect to void marker **23**) permitted the simultaneous evaluation of the binding capacity of the column (B_t) as well as the dissociation constant (K_d) via a plot similar to that depicted in Figure 3.6b. B_t was estimated to be 1300 pmol, which corresponds to an active capacity of 650 pmol of antibody B3, which has two antigen binding sites. The dissociation constant for

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[•] gift from Dr. O. Hindsgaul

compound **2** was 24 μM.

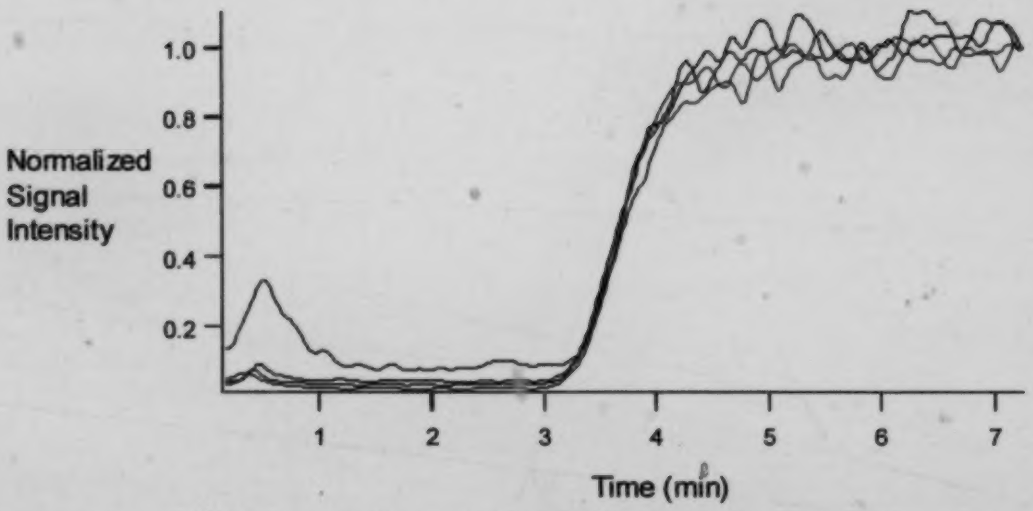


Figure 3.9. *FAC/MS assay for non-specific binding of synthetic Le^y analogs to biotinylated CPG-SA beads. The data shown here was drawn using the IGOR program. A mixture of void marker 23 (red), and Le^y analogs 2 (green), 3 (black), and 22 (blue), each present in 2 μM concentration in buffer (10 mM NH_4OAc, 1 mM NaCl, pH 7.4) was continuously infused through a blank microscale column (without immobilized B3) and the effluent was monitored. Shown here are the overlaid, extracted ion chromatograms. All compounds in the mixture broke through simultaneously, confirming the absence of specific binding.*

Having the value of K_d for Le^y pentasaccharide **2**, it was possible to screen a mixture of Le^y analogs **2**, **3**, and **22** and evaluate the two unknown dissociation constants simultaneously. A mixture of compounds **2**, **3**, and **22** were infused through the B3 affinity column and their retention volumes measured with respect to void marker compound **23** (Figure 3.10). Since the column binding activity B_t decreases slowly with time, it was necessary to re-estimate B_t using Equation 1 and the value of K_d for Le^y pentasaccharide **2** before the values of the apparent K_ds for hexasaccharide **3** and tetrasaccharide **22** could be evaluated. These values, 12 and 11 μM respectively, are designated K_d^{mix} since they are apparent K_ds measured in the presence of a mixture of ligands.

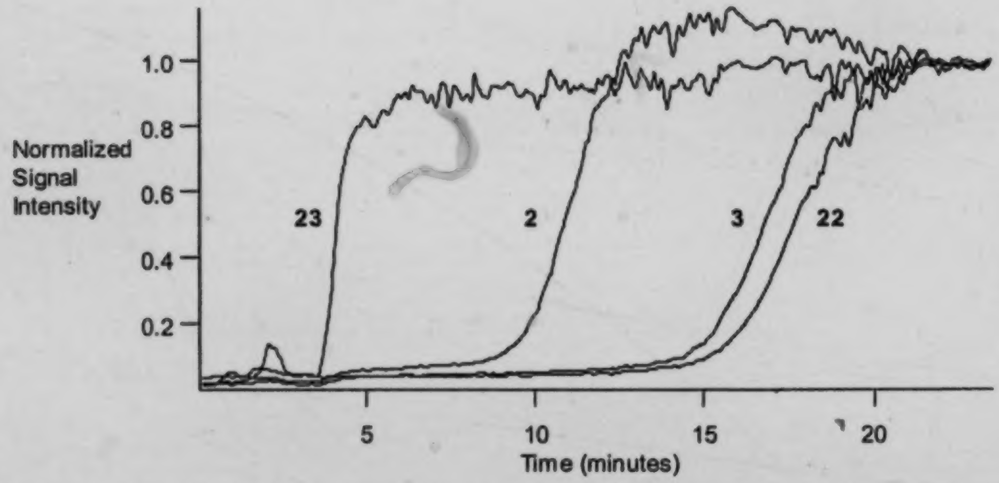


Figure 3.10. *FAC/MS screening of the activity of Le^y derivatives 2, 3, and 22 as ligands for antibody B3. The data shown here was drawn using the IGOR program. A mixture of the three Le^y derivatives and void volume marker 23 (2 μM each) in buffer (10 mM ammonium acetate, 1 mM sodium chloride, pH 7.4) was infused (8 μL/min) through a B3 affinity column and the effluent was monitored by electrospray MS in SIM and positive ion mode. The overlaid, extracted ion chromatograms for the compounds are shown here.*

The K_ds were also determined individually for hexasaccharide **3** and tetrasaccharide **22** (in the manner described for compound **2**); these values were similar (11 and 8 μM, respectively) but slightly lower than the K_d^{mix} values.

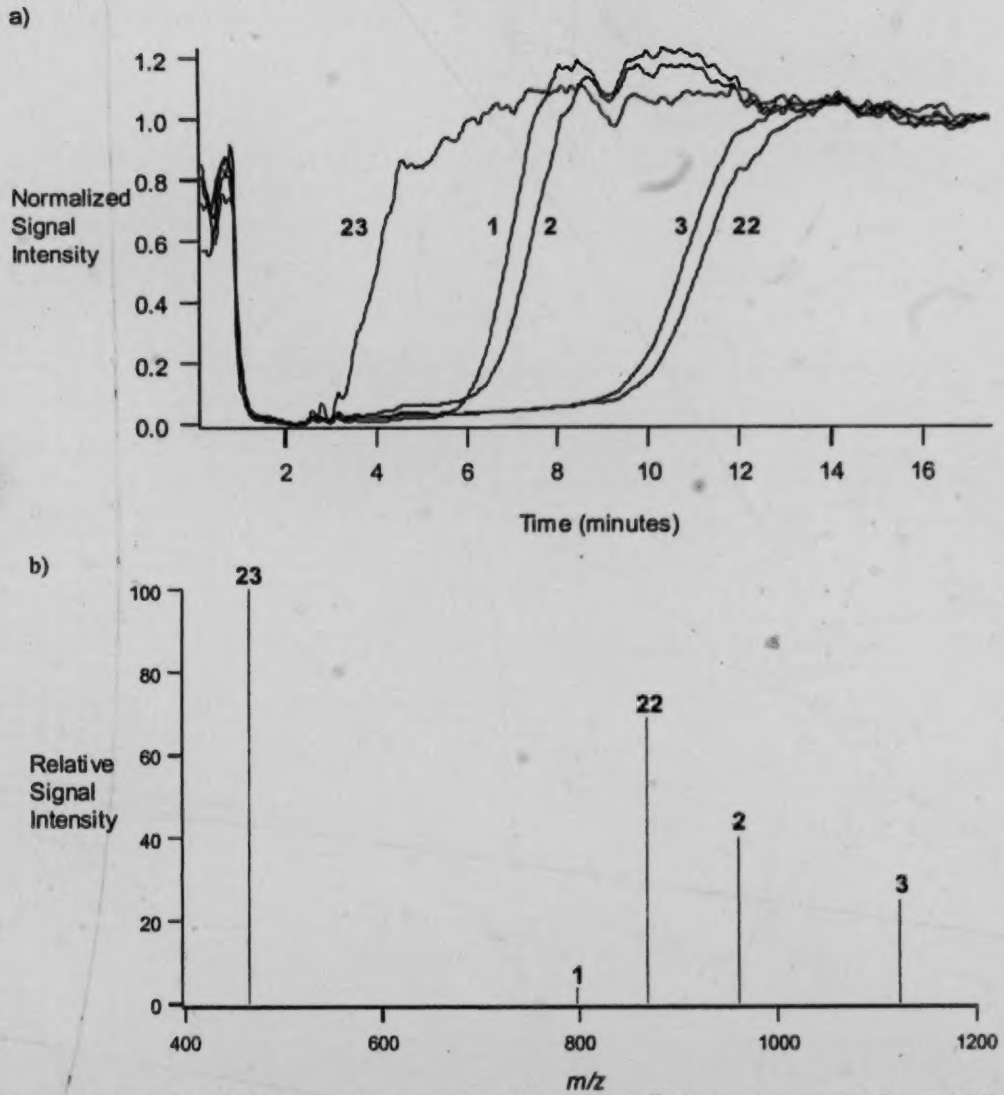


Figure 3.11. *FAC/MS screening of B3 affinity for Le^y derivatives 1, 2, 3, and 22. A mixture of the three Le^y derivatives and void volume marker 23 (2 μM each) in buffer (10 mM NH_4OAc, 1 mM NaCl, pH 7.4) was infused (8 μL/min) through a B3 affinity column and the effluent was monitored by electrospray MS in SIM and positive ion mode. (a) The overlaid, extracted ion chromatograms for the compounds, drawn using the IGOR program. (b) Mass spectrum of the effluent from the B3 affinity column after compounds 1, 2, 3, 22 and 23 have broken through at their infusion concentration. The compounds were monitored at* m/z *values corresponding to their sodium adducts, i.e., $(M + Na)^+$.*

In order to probe the nature of the enhanced binding activity exhibited by compound **22**, the relative activity of compound **1** was also evaluated in a mixture. Tetrasaccharides **1** and **22** are identical in carbohydrate structure (Le^y) but possess aglycons differing significantly in hydrophobicity and size. FAC/MS analysis of a mixture of tetrasaccharide **1**, pentasaccharide **2**, hexasaccharide **3**, and tetrasaccharide **22** indicated that compounds **1** and **2** possess similar affinity for antibody B3 and both are less active than compounds **3** and **22** (Figure 3.11). Retention volumes were measured for each compound in the mixture with respect to void volume marker **23**. Using these values, the individually-determined K_d values for **2**, **3**, and **22**, and Equation 1, the K_d^{mix} for **1** was determined to be 27 μM.

It is noteworthy that the break-through volumes for compounds **2**, **3** and **22** shown in Figure 3.11 are less than those observed for the same three compounds in the earlier FAC/MS experiment (Figure 3.10); this difference in retention by the same B3 affinity column corresponds to a 50% decrease in the binding capacity of the B3 column over 16 months (approximately 60 runs).

The relative reactivity ranking of the compounds by FAC/MS is identical to that determined by enzyme-linked immunosorbent assays (ELISAs) performed by J. Sadowska. As summarized in Table 3.1, inhibition constants (IC_{50}) were measured in two separate ELISA experiments: in one experiment, only compounds **2**, **3**, and **22** were compared; the other experiment screened only tetrasaccharide **1** and hexasaccharide **3** (Figures 3.12 and 3.13). The IC_{50} values corroborate the same relative order of binding affinity but are approximately 1-3 times higher than the K_ds measured by FAC/MS.

Table 3.1. *Summary of FAC/MS and ELISA data*

Compound	*m/z*	IC_{50} (μM)		K_d (μM)	
	(M + Na)	Exp. 1	Exp. 2	Mixture	Individual
1	798	-	34	$27 \pm 8^{**}$	-
2	960	67	-	-	24 ± 4
3	1122	21	14	12 ± 2	11.3 ± 0.5
22	868	12	-	11 ± 2	7.7 ± 0.8

FAC/MS data were measured at room temperature in either duplicate or triplicate experiments except for that marked with two asterisks (**), which was determined from a single experiment using a mixture of ligands. (See Appendix A.2 for a description of the data analysis).

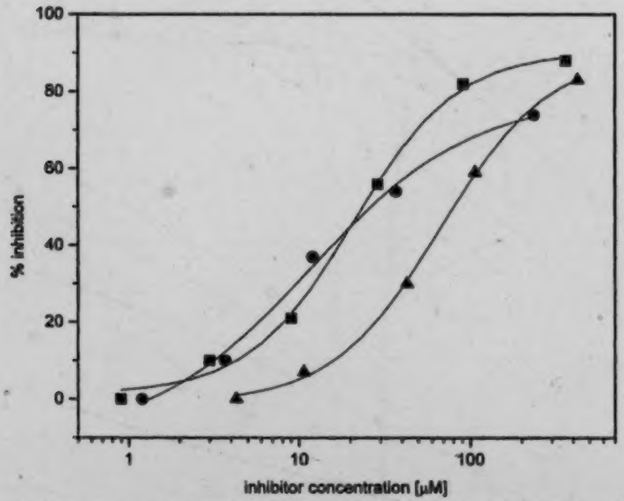


Figure 3.12. *Inhibition of B3 binding to synthetic Le^y-BSA glycoconjugate with 3 synthetic Le^y derivatives. Shown overlaid are the data obtained from inhibition ELISAs using pentasaccharide 2 (▲), hexasaccharide 3 (■), and tetrasaccharide 22 (●).*

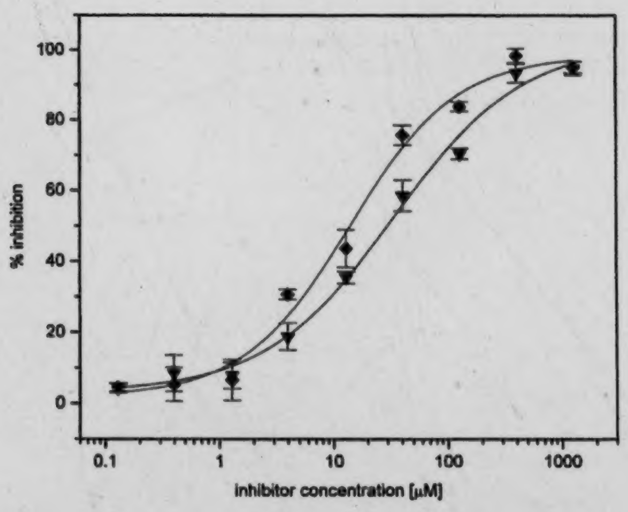


Figure 3.13. *Inhibition of B3 binding to synthetic Le^y-BSA glycoconjugate with 2 synthetic Le^y derivatives. Shown overlaid are the data obtained from inhibition ELISAs using tetrasaccharide 1 (▼) and hexasaccharide 3 (♦).*

3.3. Discussion of FAC/MS Results

The results from both FAC/MS and ELISA experiments indicate that the hexasaccharide Le^y derivative **3** and the 8-methoxycarbonyloctyl Le^y derivative **22** have greater affinity for antibody B3 than the tetrasaccharide derivative **1** or the pentasaccharide derivative **2**. The "bumps" observed in the frontal affinity chromatograms of compounds **1** and **2** when assayed in a mixture with the stronger ligands **3** and **22** (Figures 3.10 and 3.11) confirm that all the ligands are competing for the same binding site.

Since "bumps" in the frontal chromatograms arise from weak ligand displacement by competing, stronger ligands,[90] they are also an indication that the concentrations of the compounds in the assayed mixture were not sufficiently low (in comparison to their K_ds) that the breakthrough volume observed for each compound was independent of the mixture composition. That is, the value obtained for K_d^{mix} when a "bump" was observed in the chromatogram must be an overestimate of the individually determined K_d value. As summarized in Table 3.1, the K_d^{mix} values are indeed larger than the K_d values determined for each compound independently, but are still good approximations. A closer approximation of K_d by K_d^{mix} would have been possible had the mixtures been diluted and re-analyzed.

The binding affinity of B3 for the synthetic Le^y antigens (dissociation constants between 8 and 27 μM) falls within the usual range for a carbohydrate-specific antibody. For example, the dissociation constant for the interaction of the antigen-binding fragment of anti-Le^y antibody BR96 and a synthetic ligand is 5 μM.[33] Carbohydrate-binding myeloma proteins (monoclonal antibodies from myeloma cell lines) typically bind antigen with dissociation constants between 1 and 100 μM.[39]

The origin of the greater affinity of B3 for tetrasaccharide **22** compared to tetrasaccharide **1**, which possesses an identical carbohydrate moiety, is not clear. Non-specific interactions with B3 do not account for the enhanced affinity observed for tetrasaccharide **22**, because 8-methoxycarbonyloctyl glycosides **24** and **25** carry the same aglycon as Le^y derivative **22** and are not retained by the B3 affinity column (Figure 3.8). It is also unlikely that the differences in binding affinity observed among the synthetic Le^y derivatives is due to differences in the conformation of the Le^y determinant in the four compounds. To the extent that agreement in ^{1}H NMR chemical shift data is an indication of a common conformation, the solution conformation of the Le^y tetrasaccharide that constitutes derivatives **1**, **2**, **3**, and **22**[61] is essentially identical.

It seems more probable that binding of compound **22** by antibody B3 involves first recognition of the carbohydrate portion by specific interactions, and subsequent enhancement of the free energy of complexation by fortuitous, non-specific, hydrophobic interactions with the flexible, alkyl aglycon. The interactions of B3 with the 8-methoxycarbonyl chain may be similar to the those observed in the X-ray crystal structure of the Fab of antibody BR96 in complex with **22** (Figure 3.2).[33] In the case of

BR96, it was inferred that while interactions of the antibody with the 8-methoxycarbonyloctyl group of the synthetic antigen were extensive, they were not specific, because affinity maturation mutagenesis experiments identified BR96 mutants that showed increased affinity for either the synthetic antigen **22** or for tumour cells. If specific protein contacts with the synthetic glycoside **22** were the basis for the antibody's affinity for the natural tumor antigen, then mutations in the protein residues that improved binding to the synthetic antigen would have been accompanied by parallel increases in tumor affinity.

Based on the observed dissociation constants for the four synthetic Le^y derivatives, it is inferred that the binding site of B3 does indeed accommodate larger structures, since the smallest antigen **1** was the weakest binder. Further, it seems that the reducing terminal glucose moiety of hexasaccharide **3** in particular may favourably contribute to the free energy of the B3-antigen interaction. In contrast, the additional galactose residue present in derivatives **2** and **3** does not appear to contribute to the epitope recognized by B3, since pentasaccharide **2** is approximately equal in affinity to tetrasaccharide **1** and a weaker ligand than hexasaccharide **3**.

As it is known that the Le^y tetrasaccharide is both necessary and sufficient for binding,[29] it is certain that contacts between the antibody and the four hexoses of Le^y provide most (or perhaps all) of the binding energy for the recognition event involving antibody B3 and the natural antigen on tumor cells for which it is specific. As summarized in a review of carbohydrate recognition by antibody binding sites, although a typical epitope is a di- to octasaccharide, not all hexoses contribute equally to the binding energy associated with the recognition event. Indeed, it is most common for the vast majority of the energetically important and favorable protein contacts to be localized to only two to four sugar residues while the rest of the residues combined contribute the small remaining binding energy.[39]

If the glucose moiety of hexasaccharide **3** does indeed constitute part of the epitope recognized by antibody B3, its energetic contribution to binding is minor; the difference in $\Delta G^{\circ}_{298\ K}$ associated with the recognition of tetrasaccharide **1** compared to hexasaccharide **3** is only 0.5 kcal/mol. Such an energy difference, based on generalizations about carbohydrate-protein interactions,[39] would correspond to a single hydrogen bond, a weak polar contact, or a solvent-exposed hydrogen bond between the glucose moiety and the binding site of B3. However, with only the FAC/MS experiments described here, we cannot positively differentiate between merely fortuitous and truly specific interactions that enhance the affinity of the antibody for extended structures such as **3** and **22**.

Further inferences regarding the size of the epitope recognized by antibody B3 would require additional information. Future FAC/MS assays of other, larger determinants, such as trifucosyl- and extended Le^y (Figure 3.3) for reactivity with B3 might prove useful. Also, FAC/MS screening of the same set of synthetic Le^y analogs for reactivity with other antibodies would help to elucidate the origin of tumor-specificity, especially if the data for other tumor-specific (e.g. BR96), and non-tumor-specific, anti-Le^y antibodies were compared. Results from FAC/MS assays of anti-trifucosyl Le^y antibodies (e.g. antibody KH1) would also be interesting. More direct evidence of extended structure recognition would have to be obtained by other methods, such as NMR techniques, X-ray crystal structure studies of the B3-Le^y complex, or by the capture and structural elucidation of the natural antigen.

Chapter 4

Studies Toward Ley Glycoconjugate Cancer Vaccines

4.1. Introduction

4.1.a. The Prospect for Tumor Immunotherapy

The term cancer is used for a large group of diseases, all characterized by the abnormal growth of abnormal cells. Although the precise mechanisms are not understood, it is believed that the events in cancer take place in stages.

Initially, a normal cell is converted to a latent tumor cell, possibly by alteration of a cancer-associated gene that may arise from spontaneous mutation, viral infection, or exposure to carcinogenic chemicals or ionizing radiation. Following a process of promotion, which may be simply the acquisition of a second mutation in its DNA, the transformed cell then proliferates uncontrollably to form a tumor. As the tumor grows progressively larger, its bulk affects the structure and function of the adjacent normal tissue. Local invasion of the tumor into the adjacent normal tissues leads to further disruption of normal functions. Having invaded adjacent tissues, some of the malignant cells can then metastasize, i.e. break away from the primary tumor and be carried to a distant part of the body where they grow to form a secondary tumor. It is noteworthy that the genes implicated in the development of cancer appear to be normal genes with normal functions, but when altered in transformed cells, are expressed inappropriately: at the wrong time, at the wrong levels, in the wrong cell, or in an altered form.[92, 93]

At present, traditional cancer therapy consists of a combination of surgery, radiation treatment, and drug administration. Since it is possible that a single cancer cell may give rise to a malignant tumor, complete cure of cancer requires the removal or killing of all the malignant cells in the patient, i.e. not only the cells in the primary tumor, but also the circulating tumor cells in the clinically-undetectable micrometastases.

Unfortunately, ensuring that no malignant cells survive cancer therapy is problematic, because at a given time, not all tumor cells will be sensitive to treatment. For example, a treatment that exploits the higher growth rate of tumors compared to normal tissues will not kill tumor cells that happen to be resting and not replicating. Also, a portion of the population of cells in a tumor may develop resistance to the drugs used in chemotherapy. The sequential, alternating use of high-dose combinations of non-cross-resistant treatments should help, as should immune surveillance, if tumor immunity can be elicited in the patient.[93] Immunotherapy is, therefore, generally envisioned to be suitable mostly as an adjuvant treatment, used in combination with other treatments, to target micrometastases in particular.[94]

The basis for the development of cancer vaccines is the same as for infectious disease vaccines. The potential complications associated with their development are also similar, e.g. toxic effects from autoimmunity as a result of cross-reactive anti-vaccine antibodies, mechanisms of immune surveillance evasion, and the heterogeneity of the target cells[92] (e.g. in many tumors, the cells are heterogeneous in antigen expression not only within the tumor,[94] but also in different phases of development[95]). Even so, the concept of immunotherapy holds promise. Although immunization with ganglioside antigens may induce peripheral neuropathies,[96] at least some immunogenic vaccines directed at TCAs that are also normally expressed (e.g. GM2, S-Tn, Le^y) have not caused serious adverse effects in humans.[94, 97] Further, anti-cancer vaccines have been shown to successfully elicit human antibodies that react with, and mediate complement-dependent cytotoxicity of, cancer cells *in vitro*, which suggests that the immune system's tolerance for cancer can be broken.[19] More important, cancer vaccines have been shown to be protective against experimental tumors *in vivo* in rodents.[18]

4.1.b. Previously Reported Le^y-based Anti-cancer Vaccines

A number of synthetic, carbohydrate-based conjugate vaccines that are directed at the TCA Le^y have been studied by Danishefsky *et al.*[19] All of these vaccines were designed to elicit a humoral immune response and may be categorized as "monomeric conjugate vaccines", "clustered conjugate vaccines", or "multivalent conjugate vaccines".[95]

The first-generation, monomeric conjugate vaccines **26-28** (Figure 4.1a) consist of the Le^y determinant conjugated via a spacer (either an ethyl group or 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide) to a carrier protein (either bovine serum albumin or keyhole limpet hemocyanin). Pre-clinical investigations using the vaccines in mice showed that the best immune response was obtained with conjugate **27**, i.e. with the ethyl linker, keyhole limpet hemocyanin (KLH) as the carrier protein, and QS21 as the immunological adjuvant.[97] Clinical evaluation of the Le^y-KLH conjugate **27** in humans (phase I trial) demonstrated two important points: the synthetic vaccine elicited antibodies capable of reacting *in vitro* with Le^y-expressing tumor cells, and the vaccine was well tolerated with no adverse effects related to autoimmunity, a potential complication that had been irrelevant to the experiments in mice. There were some differences between the immune responses elicited by vaccine **27** in humans and in mice, the most significant being that in humans the antibody response was only modest, mostly of the IgM class, and transient in nature. The former two observations were attributed to the inability of the KLH carrier to induce T-cell help, whereas the last could not be explained by the authors.[94]

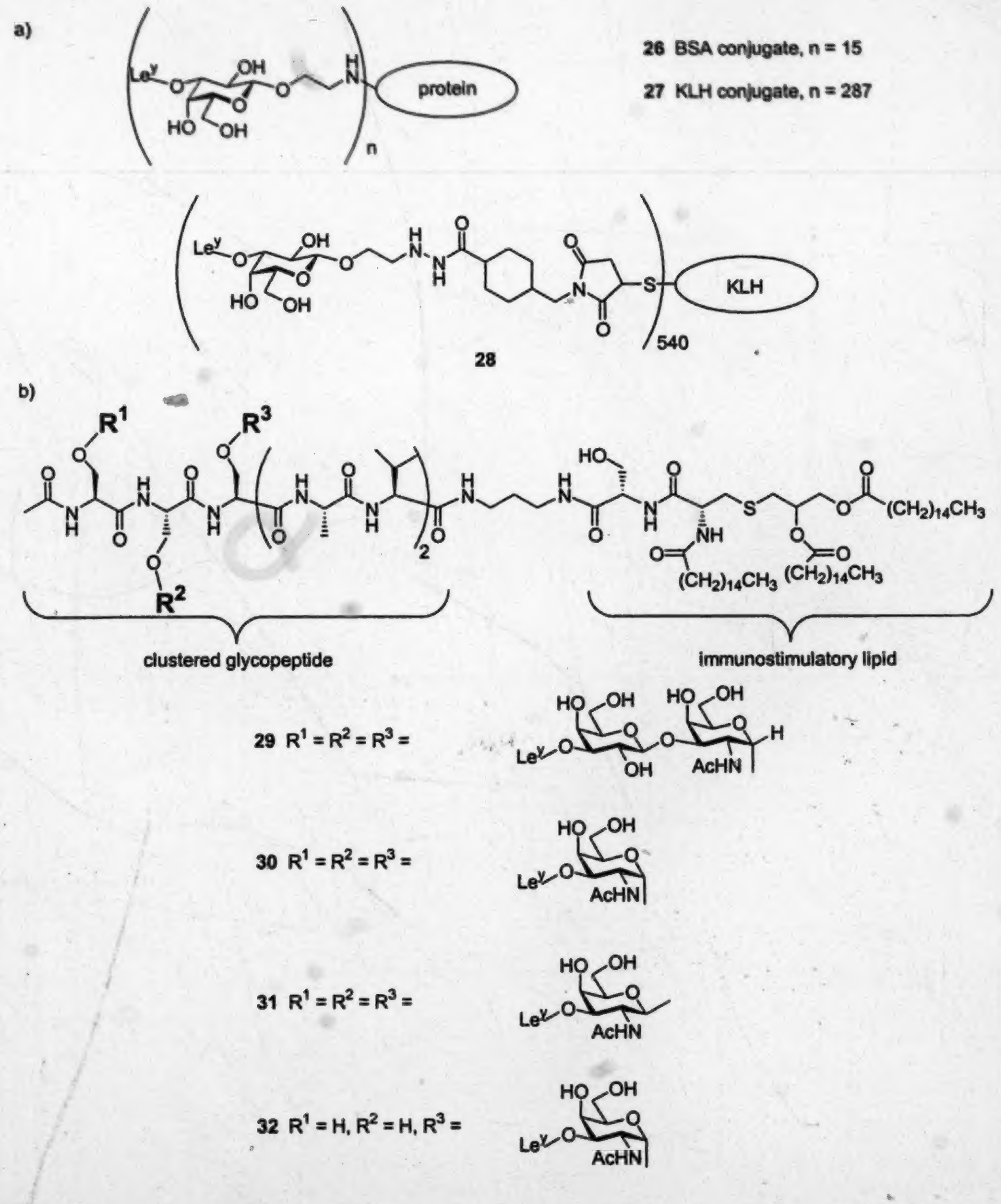

a)
Le^y
protein
n
26 BSA conjugate, n = 15
27 KLH conjugate, n = 287
KLH
540
28
b)
R^1
R^2
R^3
2
$(CH_2)_{14}CH_3$
clustered glycopeptide
immunostimulatory lipid
29 $R^1 = R^2 = R^3 =$
30 $R^1 = R^2 = R^3 =$
31 $R^1 = R^2 = R^3 =$
32 $R^1 = H, R^2 = H, R^3 =$
AcHN

Figure 4.1. *Mono-antigenic, Le^y-based anticancer vaccines synthesized by Danishefsky et al. a) Monovalent conjugate vaccines (b) Clustered conjugate vaccines. Glycolipopeptides **29-32** were designed to mimic the clustered domains of* O*-linked mucin glycoproteins.*

The second-generation, clustered conjugate vaccines **29-32** (Figure 4.1b) were constructed to mimic the presentation of the Le^y determinant in the context of a mucin glycoprotein and to more closely resemble the surface of a tumor cell.[34, 98] The two most significant differences between these conjugates and the first generation conjugate vaccines (**26-28**) are the clustered presentation of the carbohydrate antigen and the well-defined, wholly synthetic structure (important for simplifying regulatory approval for vaccines). Instead of conjugation to a carrier protein (e.g. KLH), the antigen-rich glycopeptide domain was covalently linked to an immunostimulatory lipid, tripalmitoyl-*S*-glycerylcysteinylserine. Evaluation of the immunogenicity of these vaccines with the adjuvant QS-21 in mice demonstrated that they were indeed capable of eliciting antibodies of both IgM and IgG classes that would react *in vitro* with Le^y-positive tumor cells. Comparison of the results using clustered vaccines **30** and **31** with those using the single-epitope construct **32** led to the inference that clustering is beneficial. The equal efficacy of vaccines **30** and **31** suggests that preservation of the α-*O*-linkage of the carbohydrate to the serine residue, i.e. the naturally-occurring linkage in *O*-linked glycoproteins such as mucins (Figure 3.4), is not important.[99]

In contrast to the second generation of conjugate vaccines that carried clusters of a single TCA (the Le^y determinant), the third-generation, multivalent conjugate vaccines consist of clustered displays of three different TCAs (Figure 4.2).[20, 100, 101] The third-generation conjugate vaccines were designed to elicit a broader humoral immune response against tumor cells by exploiting the fact that most tumors express multiple TCAs; a more diverse antibody response might also be helpful in addressing the problem of tumor heterogeneity. The multivalent TF-Le^y-Tn conjugate vaccine (Figure 4.2a), for example, incorporates a combination of TCAs associated with colon and prostate cancer.[100] Administration of these multi-antigenic vaccines with an immunological adjuvant (QS-21 or GPI-0100) during pre-clinical trials showed that in mice, a multivalent conjugate vaccine can induce a polyclonal antibody response against the individual antigens and that the induced antibodies can cross-react *in vitro* with antigen-expressing tumor cells. Further, the humoral response elicited against each individual antigen on the multivalent vaccine appeared to be as strong as that invoked by the corresponding monovalent conjugate vaccines.[95]

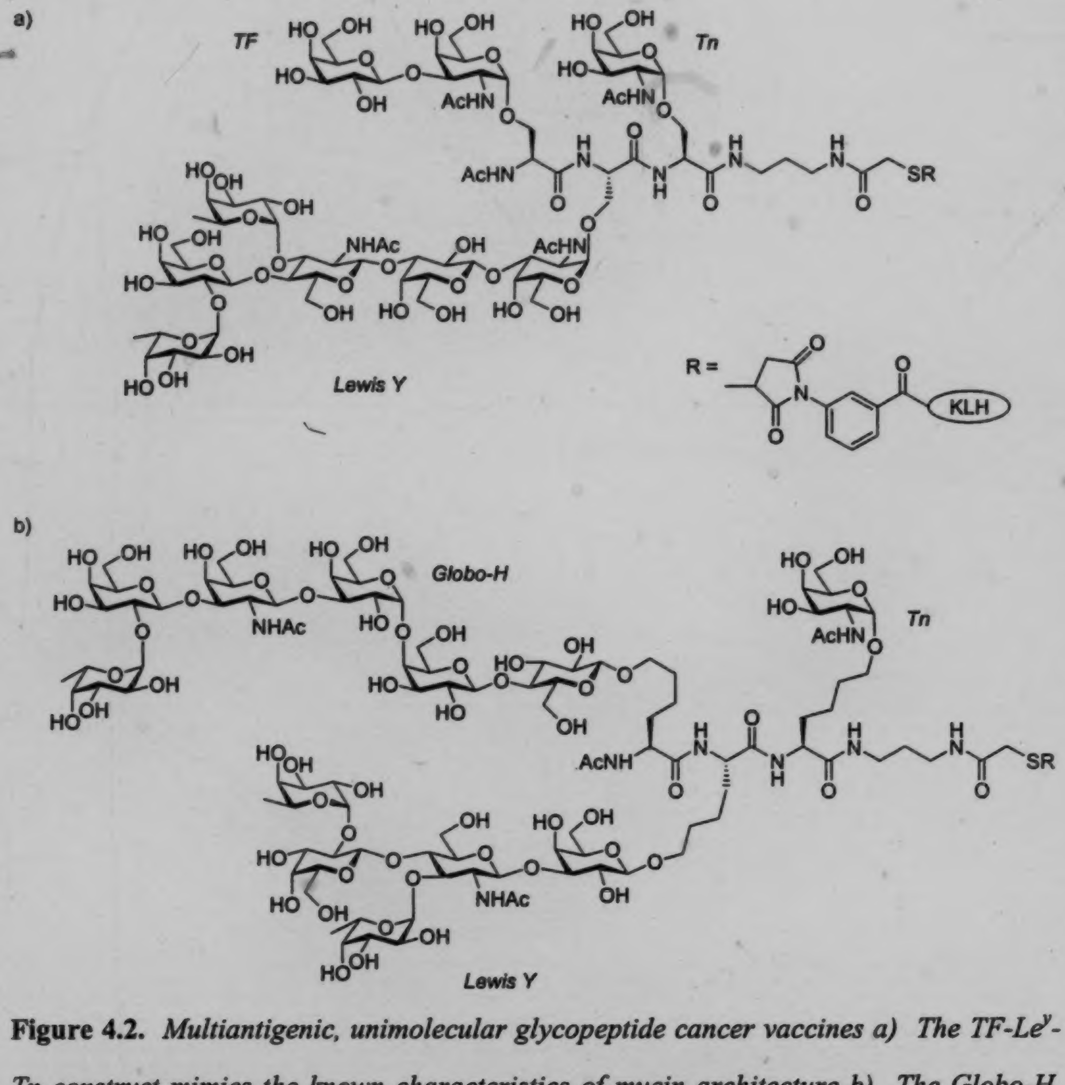


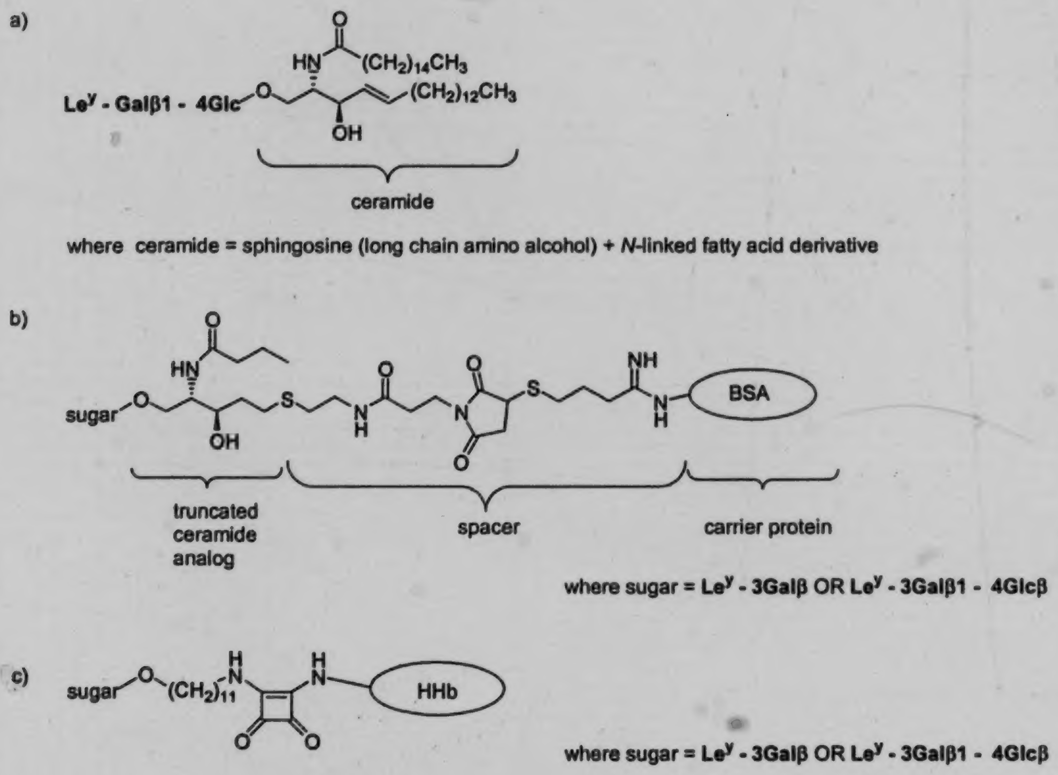
Figure 4.2. *Multiantigenic, unimolecular glycopeptide cancer vaccines a) The TF-Le^y-Tn construct mimics the known characteristics of mucin architecture b) The Globo-H, Le^y, and Tn construct does not preserve the natural α-O-linked GalNAc.*

4.2. Le^y-Protein Conjugate Vaccines: Synthetic Considerations

The immunogenicity of a conjugate vaccine is a function of many variables, each of which may exert a significant influence: the choice of protein or lipid carrier, method of coupling, use of a spacer, clustering,[19] density of antigen (ratio of antigen to carrier),[102] and the size of the antigenic determinant.[103, 104] In addition, the response elicited by the vaccine depends on properties that are not intrinsic to the conjugate itself, such as the co-administered immunological adjuvant, the immunization protocol, and the animal used.[7] Changing any of these factors may affect immunogenicity as characterized by antibody amounts (titer), specificity, affinity, isotype distribution, and booster response. Although the immunogenicity of many conjugate vaccines has been evaluated in experimental animals, and a few general guidelines have emerged from the results of studies on systematically varied vaccine analogs, vaccine design is still largely an empirical process in which the correlation between structure and immunogenicity is not always predictable.

We were interested in investigating the effect of carbohydrate antigen size on the immunogenicity of conjugate vaccines directed at the TCA Le^y. Generally, the natural antigen expressed on the membrane of a cell is larger than the epitope recognized by an antibody. However, it is not understood how large the immunizing antigen must be for optimal immunogenicity.

Specifically, we sought to evaluate the merit of conjugate vaccines bearing antigens that more closely resemble naturally occurring Le^y glycolipids. We hypothesized that such vaccines might elicit a polyclonal antibody response with greater affinity for cancer cells that express the Le^y determinant, because there is precedence for better immunogenicity stemming from more complex antigen structures.[105] Glycolipids in particular are attractive vaccine antigens for two reasons. One is the potential for glycolipid recognition by T-cells in the context of the CD1 antigen-presenting system and, as a consequence of that, the possibility of inducing both cell-mediated immunity and humoral immunity. The other is that glycolipids are better cellular targets than glycoproteins for complement-dependent cytotoxicity.[94]


a)
Le^y - Galβ1 - 4Glc
HN
(CH2)14CH3
(CH2)12CH3
OH
ceramide
b)
sugar
HN
OH
S
N H
N
S
NH
N H
BSA
truncated ceramide analog
spacer
carrier protein
c)
sugar
(CH2)11
H N
H N
HHb

where ceramide = sphingosine (long chain amino alcohol) + *N*-linked fatty acid derivative

where sugar = Le^y - 3Galβ OR Le^y - 3Galβ1 - 4Glcβ

where sugar = Le^y - 3Galβ OR Le^y - 3Galβ1 - 4Glcβ

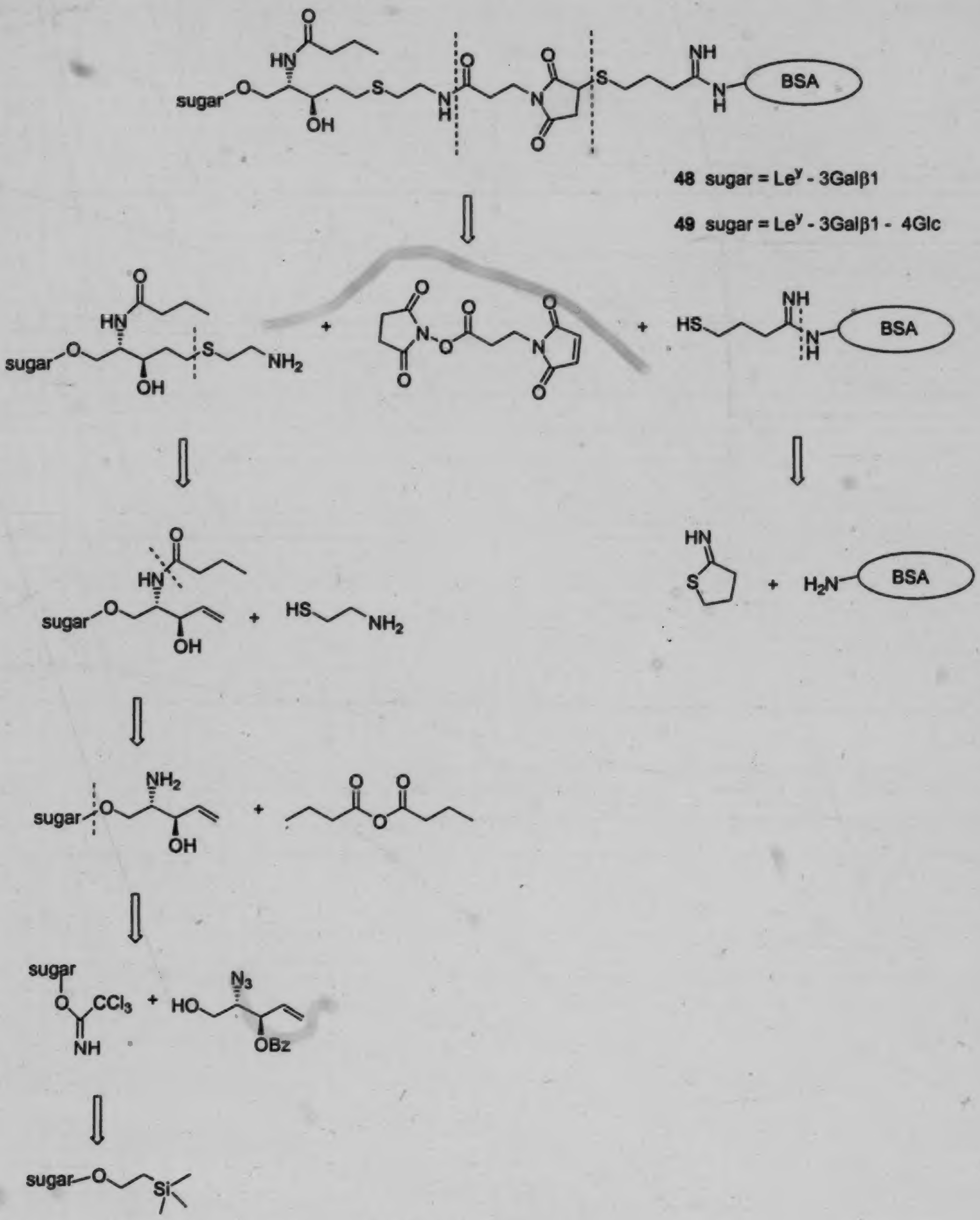
Figure 4.3. *Design of synthetic glycoconjugate targets. (a) Generalized structure of naturally occurring Le^y glycosphingolipid (b) Le^y-BSA conjugate vaccine that mimics the presentation of Le^y in the context of a glycolipid (c) Le^y-HHb conjugate for ELISA screening of carbohydrate-specific antibodies.*

Two conjugate vaccines that present the Le^y antigen in the general context of a natural glycosphingolipid were synthesized (Figure 4.3b). The pentasaccharide analog incorporates a single additional sugar (i.e. 3Gal*β*) at the reducing end of the Le^y tetrasaccharide determinant, whereas the hexasaccharide analog extends the Le^y determinant with two additional sugars (i.e. Gal*β*1-4Glc*β*, or lactose) and thereby preserves in its structure the natural linkage found in glycolipids (Figure 3.4, Figure 4.3a). A moiety resembling a ceramide was also included in the antigen; this portion includes all the stereogenic centres of a natural ceramide although the alkyl chains are truncated to reduce solubility problems during the synthesis of the glycoconjugate.

Solubility was also a consideration in the choice of carrier protein, since high solubility facilitates synthesis and purification. KLH, the carrier favored by some groups,[106] is an extremely large, multi-subunit protein that requires high salt conditions to preserve its stability and solubility and exists in several subunit aggregate states, depending on the pH.[107] To circumvent synthetic difficulty, we elected instead to use the highly-soluble, monomeric protein, bovine serum albumin (BSA). Like KLH, BSA is a commonly-used carrier protein that fulfills all the basic criteria for that purpose: it is foreign to the experimental animal (i.e. mouse), it possesses a number of functional groups that may be employed in conjugation chemistry, and it is free of glycosylation.[107]

Finally, we selected a spacer based on sulfhydryl-maleimide chemistry to covalently link the antigen and the carrier. The spacer served not only to couple the two components, but also to distance the antigen from the protein carrier and thereby facilitate steric access to the antigen. We chose to use Traut's reagent (2-iminothiolane)[108, 109] to introduce sulfhydryl groups on the carrier protein[110] followed by coupling to a maleimide-derivatized sugar,[111, 112] two strategies employed in the synthesis of other antigens and conjugate vaccines that gave relatively high yields in comparison to other methods.[97, 111]

In order to assay the polyclonal antibody response elicited by the conjugate vaccines for Le^y specificity, it was necessary to synthesize another pair of neoglycoproteins for use in indirect ELISA. For this purpose, we constructed compounds in which only the carbohydrate portion of the vaccine determinants (pentasaccharide or hexasaccharide) were conjugated via a simple alkyl linker to horse hemoglobin (Figure 4.3c).



48 sugar = Le^y - 3Galβ1

49 sugar = Le^y - 3Galβ1 - 4Glc

sugar
HN
OH
S
N
H
O
BSA
NH
+
HS
NH$_2$
N$_3$
HO
OBz
CCl$_3$
H$_2$N
Si

Figure 4.4. *Retrosynthesis of Le^y-BSA conjugate vaccines*

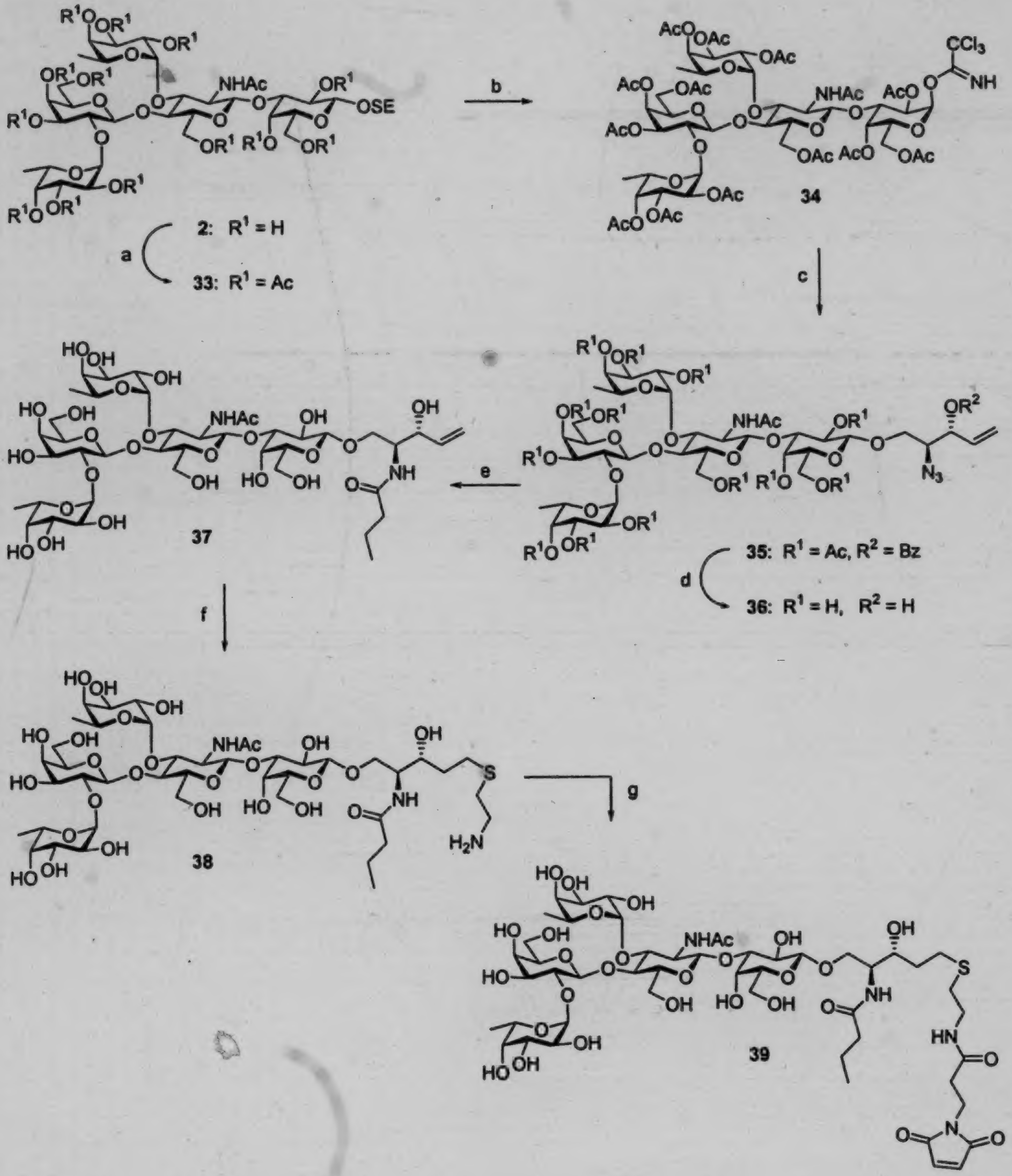
4.3. Synthesis of Le^y–BSA Conjugate Vaccines

The retrosynthesis of the penta- and hexasaccharide Le^y-BSA conjugate vaccines (**48** and **49**) is shown in Figure 4.4. It was envisioned that the conjugates could be efficiently assembled from three building blocks: an amino-functionalized, truncated glycolipid analog; a heterobifunctional cross-linker reactive to amines and sulfhydryls; and thiolated BSA. Thiolated BSA would be readily prepared by chemical modification of the accessible lysine residues of BSA with 2-iminothiolane.

The amino-derivatized antigen was disconnected into two parts: a glycolipid analog with a terminal alkene functionality and cysteamine. From here we followed the rationale of the "azidosphingosine glycosylation" strategy developed for glycosphingolipid synthesis by Schmidt *et al.*[113, 114] Thus, disconnection at the amide bond of the ceramide portion gave the corresponding sphingosine glycoside and an acylating agent; further disconnection of the sphingosine glycoside at the glycosidic bond gave two precursors: a truncated azidosphingosine acceptor and a trichloroacetimidate glycosyl donor. This linear approach reportedly gives better yields than the convergent strategy that disconnects a glycolipid into an intact ceramide moiety and glycosyl donor.[114]

The trichloroacetimidate donor would be derived from either of the 2-(trimethylsilyl)ethyl glycosides (pentasaccharide **2** or hexasaccharide **3**) that we had synthesized as ligands for antibody B3 (Chapter 2).

The synthesis of the pentasaccharide Le^y glycolipid analog in conjugatable form is shown in Scheme 4.1. 2-(Trimethylsilyl)ethyl glycoside **2** was acylated with acetic anhydride and pyridine to give the *O*-protected analog **33**. The anomeric hydroxyl was then deblocked by treatment with TFA in dichloromethane[76] to give a α/β mixture of hemiacetal anomers that were converted in good yield into glycosyl trichloroacetimidate **34** using trichloroacetonitrile and DBU.[77]


a
2: R^1 = H
33: R^1 = Ac
b
34
c
35: R^1 = Ac, R^2 = Bz
d
36: R^1 = H, R^2 = H
e
37
f
38
g
39

(a) Ac_2O, py, RT, 83%. (b) 1. TFA, CH_2Cl_2, 0°C → RT. 2. DBU, CCl_3CN, CH_2Cl_2, 0 °C, 81% over 2 steps. (c) (2S, 3R)-2-azido-3-benzoyloxy-4-penten-1-ol, 4 Å MS, $BF_3 \cdot Et_2O$, CH_2Cl_2, $(CH_3)_3CCN$, 0 °C → RT, 68%. (d) $NaOCH_3$, CH_3OH, 80%. (e) 1. H_2S, py, H_2O, Et_3N, 0 °C. 2. butyric anhydride, CH_3OH, 0 °C → RT, 44% over 2 steps. (f) cysteamine hydrochloride, AAPH, H_2O, 60 °C, 33%. (g) 3-maleimidopropionic acid N-hydroxysuccinimide ester, DMF, PBS pH 7.2, RT, 81%.

Scheme 4.1. *Synthesis of a conjugatable, pentasaccharide Le^y ceramide analog.*

Boron trifluoride diethyl etherate-promoted *β*-glycosidation of trichloroacetimidate donor **34** with (2*S*,3*R*)-2-azido-3-benzoyloxy-4-penten-1-ol* (a truncated, synthetic azidosphingosine analog) in the presence of 4 Å MS furnished glycoside **35** in moderate yield. Transesterification of glycoside **35** with catalytic sodium methoxide in methanol cleaved all the *O*-acetyl and *O*-benzoyl protective groups to give the *N*-protected glycoside **36**. Azide **36** in aqueous pyridine and triethylamine was reduced with hydrogen sulfide gas[115] and the resulting amine was *N*-acylated with butyric anhydride to give glycosyl ceramide analog **37** in ca. 44% yield, as estimated from the NMR spectrum, which showed the presence of non-carbohydrate impurities that we were unable to remove by chromatography. The free-radical addition of cysteamine to alkene **37** in water using the water-soluble radical initiator AAPH[116, 117] gave amine **38** in ca. 33% yield; this yield, though low, was still an improvement over our attempts to use UV light[118] or AIBN[119] in methanol to initiate the reaction. Despite repeated attempts to purify amine **38** by size-exclusion chromatography and reverse-phase HPLC, only excess cysteamine reagent could be separated from the product while small quantities (less than 20% as estimated from 1D ^{1}H NMR data) of other impurities could not be separated. Amine **38** was subsequently acylated with 3-maleimidopropionic acid NHS-ester to give the thiol-reactive glycosphingolipid analog **39**.

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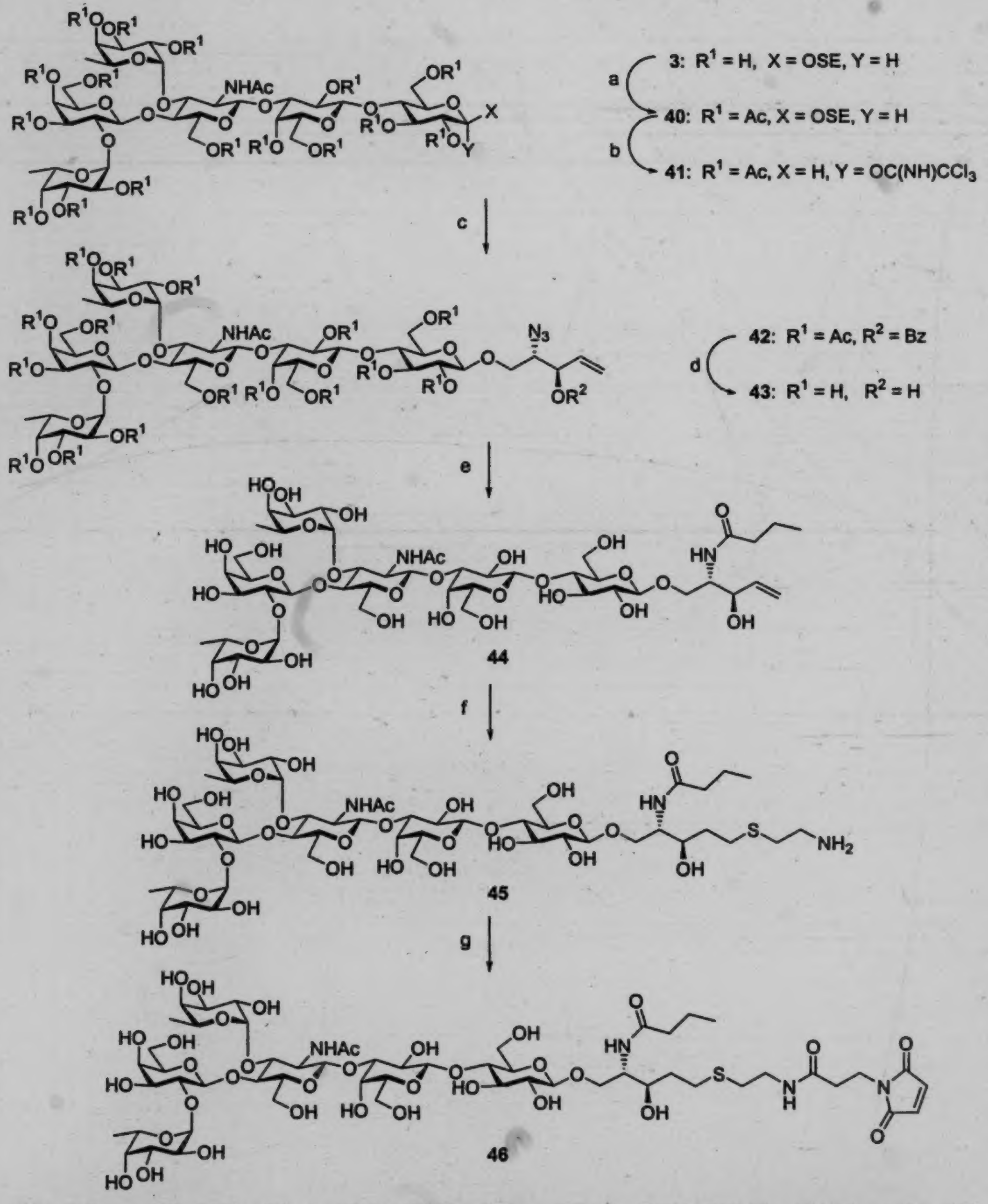
* gift from Dr. P. Zhang and C. Nycholat


a
b
3: $R^1 = H$, X = OSE, Y = H
40: $R^1 = Ac$, X = OSE, Y = H
41: $R^1 = Ac$, X = H, Y = $OC(NH)CCl_3$
c
42: $R^1 = Ac$, $R^2 = Bz$
43: $R^1 = H$, $R^2 = H$
d
e
44
f
45
g
46

(a) Ac_2O, py, RT, 68%. (b) 1. TFA, CH_2Cl_2, 0°C → RT. 2. DBU, CCl_3CN, CH_2Cl_2, 0 °C, 78% over 2 steps. (c) (2S, 3R)-2-azido-3-benzoyloxy-4-penten-1-ol, 4 Å MS, $BF_3{\cdot}Et_2O$, CH_2Cl_2, $(CH_3)_3CCN$, 0 °C → RT, 46%. (d) $NaOCH_3$, CH_3OH, 26%. (e) 1. H_2S, py, H_2O, Et_3N, 0 °C. 2. butyric anhydride, CH_3OH, 0 °C → RT, 88% over 2 steps. (f) cysteamine hydrochloride, AAPH, H_2O, 60 °C, 23%. (g) 3-maleimidopropionic acid N-hydroxysuccinimide ester, DMF, PBS pH 7.2, RT, 54%.

Scheme 4.2. *Synthesis of a conjugatable, hexasaccharide Le^y ceramide analog.*

The maleimide-derivatized hexasaccharide analog **46** was synthesized via a route analogous to the synthesis of pentasaccharide **39** (Scheme 4.2). As before, the starting material was the 2-(trimethylsilyl)ethyl glycoside (**3**). Blocking of the hydroxyls with acetyl groups gave protected glycoside **40** which was then converted in good yield (78%) from the 2-(trimethylsilyl)ethyl glycoside into the trichloroacetimidate donor **41** in two steps via the corresponding reducing sugar. Trichloroacetimidate **41**, bearing an acyl participating group at *O*-2 to favour *β*-selectivity, was glycosidated in 46% yield with the azidosphingosine analog (2*S*, 3*R*)-2-azido-3-benzoyloxy-4-penten-1-ol[*] in the presence of boron trifluoride diethyl etherate and 4Å MS. Cleavage of all the *O*-acyl protective groups of glycoside **42** with catalytic sodium methoxide in methanol afforded deblocked hexasaccharide **43** in only 26% yield, most likely due to losses during reverse-phase HPLC purification. Conversion of the azido group of glycoside **43** to an amino group using hydrogen sulfide was followed by treatment with butyric anhydride in methanol to furnish the ceramide analog **44** in 88% yield over the two steps. Finally, incorporation of a spacer for conjugation to BSA was accomplished in two steps: addition of an amino functionality with cysteamine hydrochloride to give amine **45** and subsequent *N*-acylation with a NHS ester-activated cross-linker to give maleimide **46**. As with the pentasaccharide analog, attempts to purify amine **45** by size exclusion chromatography and reverse phase HPLC were unsuccessful; the approximate yield was 23%. Similarly, we were unable to purify maleimide derivative **46** by reverse phase HPLC.

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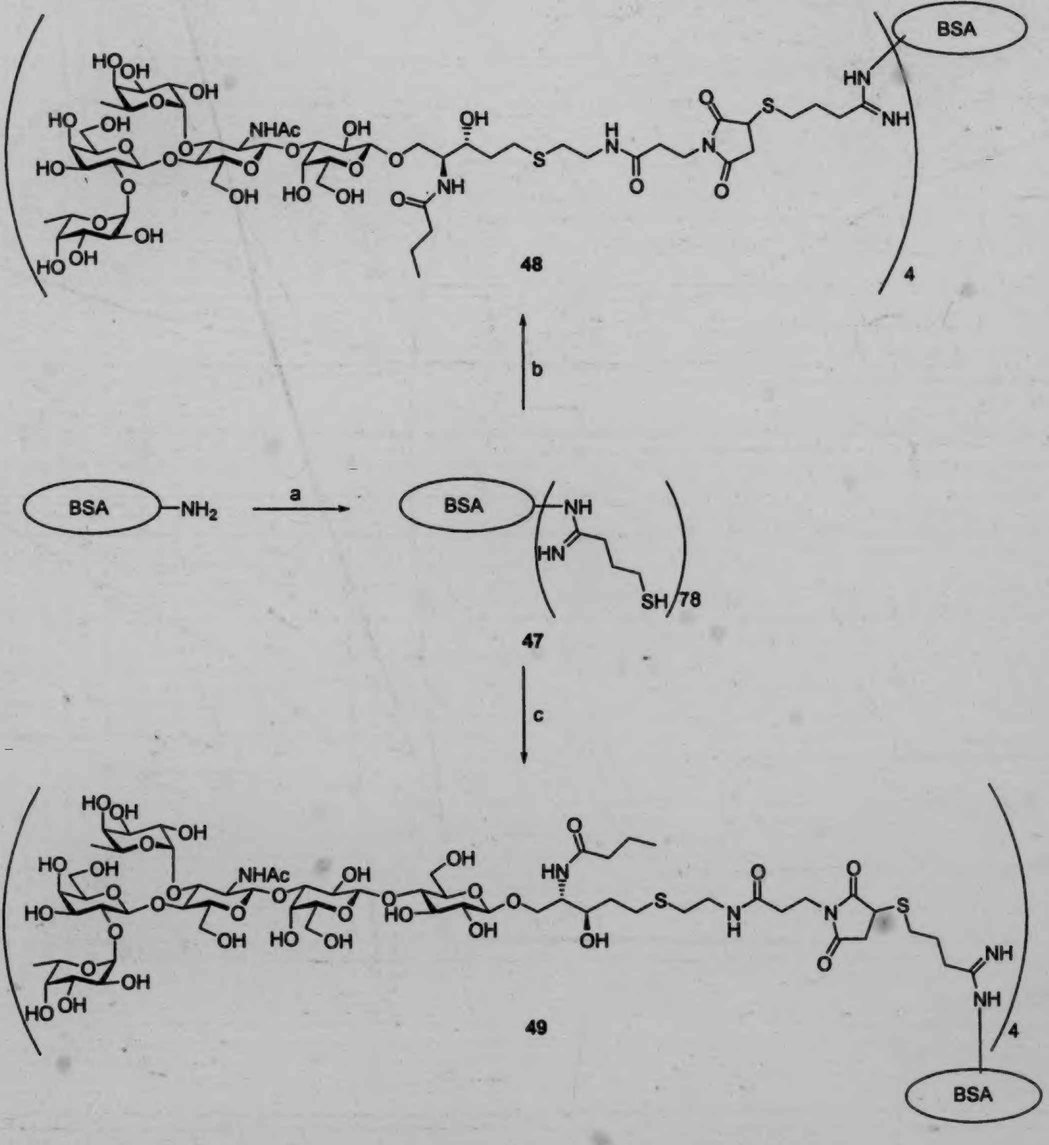
[*] gift from Dr. P. Zhang and C. Nycholat



(a) 2-iminothiolane, PBS pH 8.0 (b) **39**, PBS pH 7.2, RT (c) **46**, PBS pH 7.2, RT.

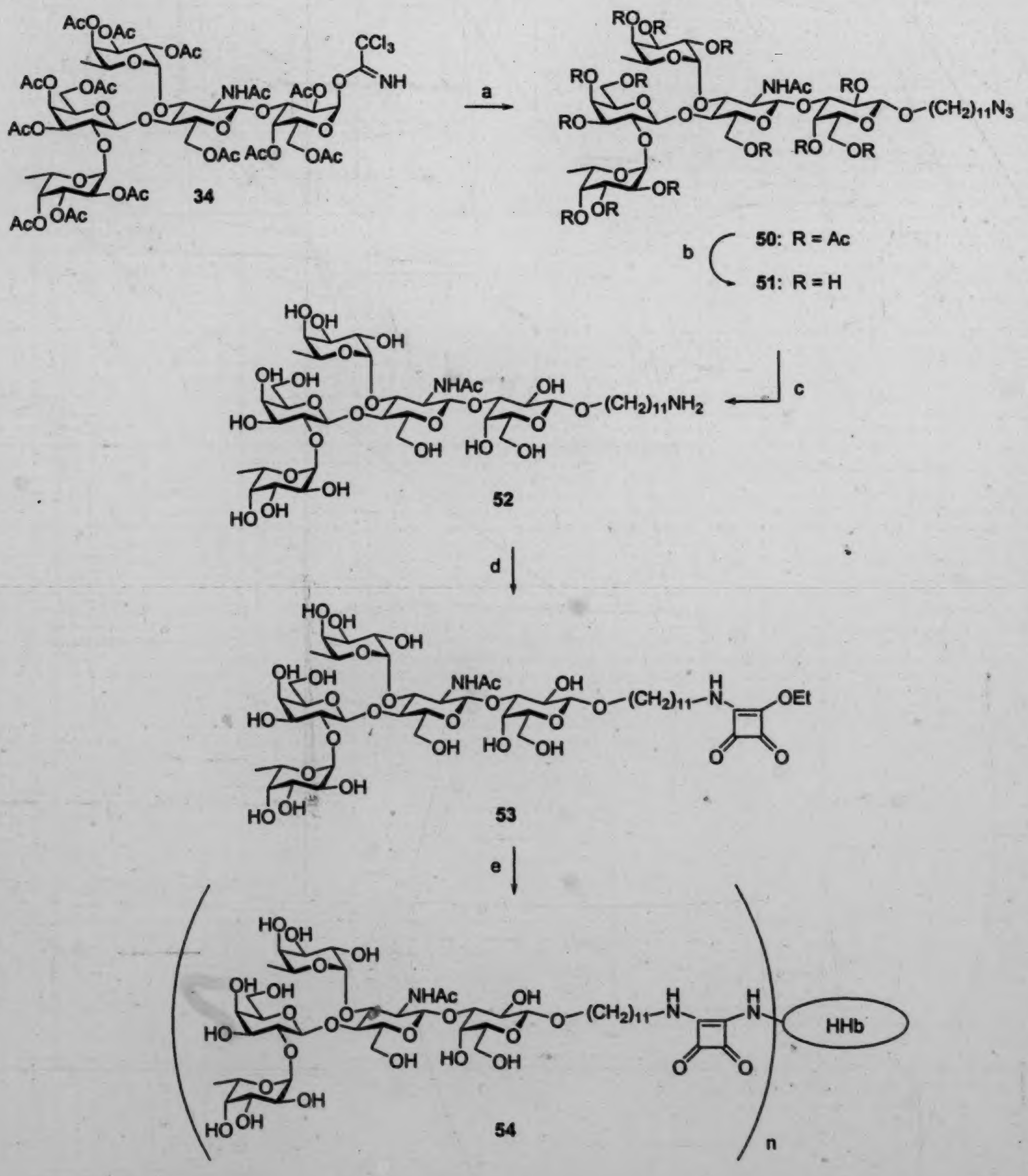
Scheme 4.3. *Synthesis of Le^y-BSA conjugates*

Having synthesized the penta- and hexasaccharide maleimide derivatives 39 and 46, construction of the BSA conjugate vaccines was straightforward (Scheme 4.3). Treatment of BSA with 2-iminothiolane resulted in thiolated BSA 47 that was characterized by MALDI mass spectrometry. The change in mass was consistent with the incorporation of 78 sulfhydryls per molecule of BSA. However, such a high efficiency of alkylation is unlikely, since BSA has only 59 lysine residues.[107] Therefore, it seems probable that some 2-iminothiolane residues were incorporated via disulfide bonds to a number of the newly-introduced sulfhydryls on BSA and that there are actually fewer than 78 reactive sulfhydryls in BSA derivative **47**.

Thiolated BSA **47** was coupled to either the pentasaccharide glycolipid analog **39** to afford conjugate **48** or to the hexasaccharide glycolipid analog **46** to give conjugate **49**. MALDI mass spectral analysis of the conjugates (Appendix A.4) indicated the addition of approximately 4 carbohydrate residues per molecule of thiolated BSA, which corresponds to a coupling yield of 27-29% with respect to the carbohydrate starting materials, **39** and **46**. This is much lower than the yield (87%) we observed in our studies using the analogous lactose (disaccharide) derivatives as a model compound (data not shown), and also lower than the yield (50%) reported for a similar coupling using a tetrasaccharide Le^y antigen.[111] Though disappointing, this is not completely unexpected, since a trend of poorer coupling efficiency with increasingly large and branched carbohydrates has been reported before.[111]

4.4. Synthesis of Le^y-HHb Conjugates for ELISA Assays

One of the methods by which we planned to evaluate the immunogenicity of the synthetic Le^y-BSA conjugate vaccines was indirect ELISA. For experiments that assess the carbohydrate specificity of the induced polyclonal antibody response, we required glycoconjugates bearing the same carbohydrate structures used in the immunogens but possessing a different spacer and a different protein carrier. We chose the unbranched undecyl group as the spacer, and horse hemoglobin (HHb) as the protein carrier. HHb is an unglycosylated protein that has been successfully used before as a carrier for carbohydrate antigens in ELISA assays of polyclonal antibodies raised in mice and rabbits against BSA glycoconjugate vaccines.[105]


34
a
50: R = Ac
51: R = H
b
c
52
d
53
e
54
HHb
n

(a) 11-azido-undecanol, 4 Å MS, CH_2Cl_2, $(CH_3)_3CCN$, $BF_3{\cdot}Et_2O$, 0 °C → RT, 47%. (b) $NaOCH_3$, CH_3OH, 32%. (c) H_2S, py, Et_3N, H_2O, 0 °C, 33%. (d) diethyl squarate, PBS pH 7.2, RT. (e) horse hemoglobin, borate buffer pH 9.0, RT.

Scheme 4.4. *Synthesis of a pentasaccharide Le^y–HHb conjugate for ELISA screening of mouse sera.*

Schemes 4.4 and 4.5 outline the assembly of the penta- and hexasaccharide glycoconjugates for ELISA assays. The synthetic routes were analogous at every step. 11-Azido-undecanol[*] was glycosylated with either the pentasaccharide (**34**) or hexasaccharide (**41**) trichloroacetimidate donor in the presence of boron trifluoride-diethyl ether and 4 Å MS in low yield (47% and 16%, respectively) to give the corresponding glycosides **50** and **55**. The trichloroacetimidate donor (**34** or **41**) had been derived in three steps from the corresponding 2-(trimethylsilyl)ethyl glycoside, as shown before in Schemes 4.1 and 4.2.

Transesterification of the per-*O*-acetylated glycoside (**50** or **55**) with sodium methoxide gave the deblocked glycoside (**51** or **56**) that was subsequently reduced with hydrogen sulfide to unmask the primary amine functionality (**52** or **57**) on the linker. The amino-derivatized carbohydrate antigen was then coupled to the lysine residues of HHb via the homobifunctional linker, diethyl squarate[120, 121] to give the target glycoconjugate (**54** or **59**). The conjugation was carried out in two steps: initial coupling of the carbohydrate moiety to the squarate linker to give the mono-ester (**53** or **58**) at pH 7.2 and then coupling to HHb at pH 9.0. Confirmation of successful coupling between the carbohydrate antigen and the protein was via MALDI mass spectral analysis of the final product (**54** or **59**) compared to the unmodified HHb. Although a change in mass was observed, estimation of the incorporation of carbohydrate residues is difficult because HHb dissociates into two pairs of subunits with similar, but not identical, mass (Appendix A.4).

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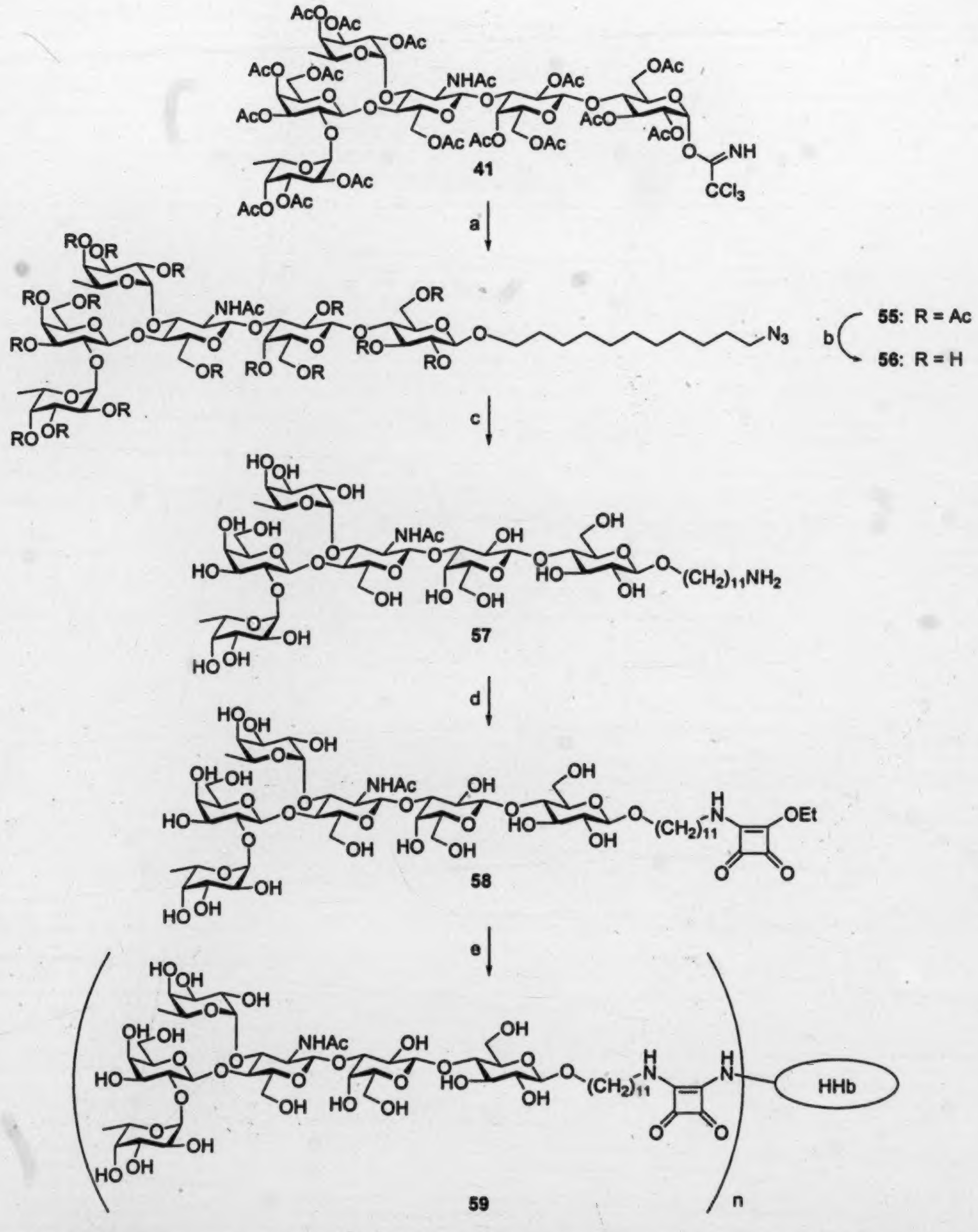
[*] gift from Dr. S. Andersen


41
a
55: R = Ac
b
56: R = H
c
57
d
58
e
HHb
59
n

(a) 11-azido-undecanol, 4 Å MS, CH_2Cl_2, $BF_3 \cdot Et_2O$, 0 °C, 16%. (b) $NaOCH_3$, CH_3OH, quant. by TLC. (c) H_2S, py, Et_3N, H_2O, 0 °C (d) diethyl squarate, PBS pH 7.2, RT. (e) horse hemoglobin, borate buffer pH 9.0, RT.

Scheme 4.5. *Synthesis of a hexasaccharide Le^y–HHb conjugate for ELISA screening of mouse sera.*

For both the penta- and hexasaccharide analogs, the yields obtained in the synthetic sequence were generally low, due in part to the steric bulk of the molecules and complications arising from having only very limited quantities with which to work; however, there is certainly opportunity for improvement and the synthesis should be optimized if it is attempted again.

4.5. Immunological Evaluation of Le^y-BSA Vaccines

The experiments carried out to evaluate the immunogenicity in BALB/c mice of the penta- and hexasaccharide Le^y-BSA conjugate vaccines (**48** and **49**) were performed by J. Sadowska.

Although the results from trials in a mouse model cannot be extrapolated to accurately predict the response induced in humans,[94] proof of immunogenicity in an experimental animal is a prerequisite to clinical evaluation. Using ELISA assays, we wanted to evaluate and compare the humoral response elicited by each conjugate for the following parameters: the titer of polyclonal, carbohydrate-specific antibodies elicited; the immunoglobulin class (i.e. IgM or IgG) of the antibody response; the reactivity of the polyclonal antibodies with cancer cells over-expressing Le^y (cell line MCF-7); and the reactivity of the polyclonal antibodies with cells that express the Le^y determinant only sparingly (cell line SKMEL).

The preliminary ELISA assays on the pre-immunized mouse sera for reactivity with the Le^y-HHb conjugates were designed to serve as a negative control, i.e. demonstrate that the mouse sera do not contain anti-Le^y antibodies prior to immunization and that any post-immunization changes in sera reactivity observed using the same assay format may be attributed to induction by the synthetic conjugate vaccines.

Regrettably, the results from these preliminary assays indicated that the pre-immunized mice already possessed antibodies that react with the synthetic HHb glycoconjugates (**54** and **59**) used in the ELISA screen. Attempts to deconvolute the apparent affinity of the pre-immune sera for the synthetic glycoconjugates and identify the cross-reactive structure (e.g. protein antigen or carbohydrate antigen) have given only ambiguous and inconsistent results thus far. Also unfortunate is that the post-immunization mouse sera do not exhibit a boosted response against the synthetic glycoconjugates used in the ELISA screen when compared to the pre-immunized mouse sera; this is an indication that the BSA glycoconjugates **48** and **49** were not immunogenic in the BALB/c strain of mice. This is a surprising result since BALB/c mice are known to respond to the Le^x antigen and several reported monclonal antibodies to Le^y have been generated in BALB/c mice.[21, 23, 29] In contrast it is also known that the kidneys of BALB/c mice express Le^x antigen and it is consequently an auto-antigen.

Further immunological evaluation must be postponed until these preliminary results are understood and may require immunization of a different strain of mice.

Chapter 5

Conclusions

We successfully synthesized three 2-(trimethylsilyl)ethyl glycosides (**1**, **2** and **3**) incorporating the Le^y motif as ligands to probe the epitope recognized by anti-Le^y monoclonal antibody B3. We also synthesized four Le^y-based protein conjugates for immunological evaluation as cancer vaccines (i.e. **48** and **49**) and for ELISA screening of anti-Le^y sera (i.e. **54** and **59**). With respect to efficiency, the synthetic strategies we employed in the assembly of the carbohydrate domains compare well with routes to similar Le^y derivatives reported by other groups.[57, 63, 65, 66] Though our strategies were unique, the methods we used to establish key linkages in our constructs were drawn from previously described advances in carbohydrate and glycolipid synthesis. In particular, thioglycoside[122] and trichloroacetimidate[77] donors proved to be useful intermediates, and the Koenigs-Knorr glycosylation,[76] halide-ion catalyzed glycosidation,[85] and the azidosphingosine glycosylation[114] methods were most valuable for the formation of critical glycosidic bonds. Subsequent conjugation of the glycolipid domain to protein in the synthesis of the vaccine candidates **48** and **49** relied on diethyl squarate chemistry and sulfhydryl-maleimide chemistry, two established bio-conjugation techniques[107] that were reasonably effective in our hands.

We had set out to use our synthetic Le^y analogs and conjugates to explore the fine specificity of antibodies that are tumor-specific. It seemed likely that the higher tumor-selectivity exhibited by some antibodies, compared to others that nominally bind the same carbohydrate determinant, is due to recognition of a larger, more complex epitope. Related to this, we were also interested in investigating whether immunization with vaccine candidates carrying more complex antigenic structures would generate a more tumor-selective humoral immune response than immunization with vaccine candidates bearing smaller, minimal determinants.

Using FAC/MS and synthetic Le^y glycosides (**1**, **2**, **3**, and **22**), we demonstrated that B3, a monoclonal antibody that shares structural characteristics with other anti-Le^y monoclonal antibodies that are notable for their selective tumor reactivity (e.g. BR96 and BR55-2),[32] does indeed bind with greater affinity structures that extend the Le^y antigen at the reducing end. The difference in the K$_d$s estimated for the hexasaccharide Le^y analog **3** and the tetrasaccharide Le^y determinant **1** is small (ca. two-fold), but possibly important *in vivo* where the antibody would be reacting with a multivalent presentation of the natural, extended antigen on a tumor cell. Further inferences require that more data be obtained using other, related antibodies (e.g. BR96 and BR55-2).

Whereas our FAC/MS studies using B3 corroborate the proposal that the origin of tumor selectivity may be recognition of a larger, more complex epitope, the immunological study aimed at investigating the merits of immunizing with more complex antigens was less satisfying because we were unable to make any useful inferences from the results we obtained to date. The two vaccine candidates we synthesized, which differ from previously reported Le^y-based vaccines[19] by targeting glycolipid antigens in particular, appear to lack immunogenicity in BALB/c mice (or at least in the mature retired breeders we were obliged to use for the experiment). Also perplexing was the apparent reactivity of pre-immunized mouse sera with the Le^y-HHb conjugates used in ELISA screening.

The immunological results were surprising because there have been several reports of Le^y-reactive antibodies elicited in BALB/c mice. Further, though BALB/c mice are known to possess naturally occurring anti-Le^x antibodies,[123] the extent of cross-reactivity with the Le^y determinant that we observed in the ELISA screening of pre-immunized mouse sera has not previously been reported. Aside from possible problems inherent to the use of BALB/c mice as an experimental animal, the lack of immunogenicity may be intrinsic to the vaccine composition itself, but possible reasons for this are also not clear. Kitamura *et al.* have reported immunological studies using a synthetic Le^y-BSA conjugate in BALB/c mice, thus demonstrating that BSA as a carrier protein is not problematic *per se.*[21] While the ratio of carbohydrate antigen to carrier protein in our vaccine constructs is relatively low (4:1) compared to the density of antigens on the BSA conjugate evaluated by Kudryashov *et al.* (30:1),[97] a study on the influence of hapten density on the immunological properties of hapten-BSA conjugates demonstrated that a hapten density of 5:1 can be immunogenic.[102] Even so, we cannot rule out that low hapten density may be the origin of the poor immunogenicity we observed, because the latter study was carried out using a non-carbohydrate hapten in F1 mice[102] and the results of that study may not apply directly to the case at hand.

Glunz *et al.* have remarked that historically some synthetic constructs are both immunogenic and productively antigenic, whereas others are not.[34] The explanation for this cannot be stated with certainty on the basis of our current understanding of immunology and tumor biology. It is when we are confronted with puzzling results such as this that the gaps in our understanding of the biology behind tumor immunotherapy are manifested and it is evident that one of the remaining obstacles to the therapeutic use of cancer vaccines is our lack of knowledge with respect to the optimal choice of antigen, delivery, adjuvant, and administration.[28]

Further research in the area of carbohydrate anti-cancer vaccines is required not only to identify optimal vaccine structure and components, but also to address other major obstacles in tumor immunotherapy: the heterogeneity of tumor cells, the genetically based heterogeneity of the immune response,[18] and the possibility of the selection and overgrowth of tumor cells capable of escaping immunological surveillance by actively induced immunological pressure (analogous to the selection and overgrowth of drug-resistant tumor cells by chemotherapy).[7, 28, 93]

Chapter 6

Experimental

6.1. Synthesis

6.1.a. Reagents

(2*S*,3*R*)-2-azido-3-benzoyloxy-4-penten-1-ol and 11-azido-undecanol were synthesized and were obtained from Dr. P. Zhang, C. Nycholat, and Dr. S. Andersen. Unless otherwise specified, all commercial reagents were used as supplied. Solvents that are described as "dry" were distilled from still pots containing appropriate drying agents. Aqueous solutions for work-up procedures were made from deionized water; aqueous solutions for reactions or chromatography were made from MilliQ water. Crushed molecular sieves were activated for use by flaming in a round bottom flask under vacuum. Dowex 502-X12 cation exchange resin (ionic form H^+) was from J. T. Baker and was washed with methanol prior to use.

6.1.b. Chromatography

Analytical thin layer chromatography (TLC) was performed on silica gel 60-F_{254} from EM Science/Merck. TLC detection was achieved by fluorescence quenching under UV light, charring with 5% sulphuric acid in ethanol, or staining with cerium molybdate. Column chromatography was performed on silica gel 60 (230-400 mesh) from SiliCycle or Rose Scientific. Sep-Pak (C-18) cartridges were from Waters. High performance liquid chromatography (HPLC) was performed using a Waters HPLC system on a Beckman 5 μm C-18 (10 mm X 250 mm) column, Phenomenex 5 μm C-18 (10 X 250 mm) column, or Phenomenex 5 μm C-8 (10 X 250 mm) column. The HPLC effluent was monitored with a UV detector at 216 nm.

6.1.c. Spectral Analysis

Optical rotations were measured on a Perkin Elmer 241 polarimeter at 22 °C. Microanalyses, mass spectra, and some optical rotation measurements were obtained from the technical staff in the Departmental mass spectrometry, elemental analysis, and spectral service laboratories.

^{1}H NMR spectra (1D, COSY, TOCSY, and TROESY) were acquired on 400, 500, or 600 MHz Varian spectrometers. ^{13}C NMR spectra were acquired on 500 or 600 MHz Varian spectrometers. All experiments were performed at 27 °C. Proton chemical shifts are referenced to an internal standard at δ 7.24 ppm for solutions in $CDCl_3$, δ 3.30 ppm for solutions in CD_3OD, or to 0.1% external acetone at δ 2.225 ppm for solutions in D_2O. Carbon-13 chemical shifts are referenced to an internal standard at 77.0 ppm for solutions in $CDCl_3$, 49.0 ppm for solutions in CD_3OD, or to 1% external acetone at 31.07 ppm for solutions in D_2O. In most cases, carbon-13 NMR data was obtained from APT experiments performed on a 500 MHz spectrometer. For compounds **9**, **13**, **20**, **37**, **38**, **44**, **45**, **51**, and **52**, ^{13}C NMR data were obtained from HMQC experiments performed on either a 500 MHz or a 600 MHz spectrometer.

Sugar residues are designated with prime marks (') in the order that the residue name appears in the compound name; the sugar named first has the most prime marks. When there are non-equivalent geminal protons, they are arbitrarily called "a" and "b". In cases where the aglycon is highly functionalized, protons are designated with capital letters (Figure 6.1) for clarity.

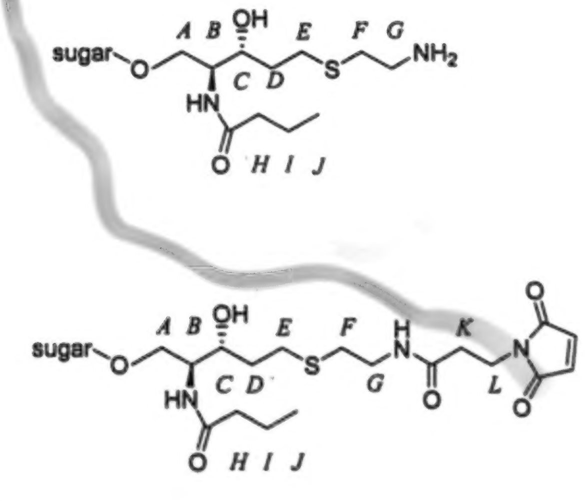


Figure 6.1. *Labelling of aglycon protons used in NMR signal assignments*

6.1.d. Products

In instances when repeated attempts at chromatographic separation failed to furnish a pure product, an estimate of the purity of the mixture was made based on 1D ^{1}H NMR data. Quantities of impurities were deemed "small" if the impurities were estimated to be less than 20% of the mixture (by mass).

2-(Trimethylsilyl)ethyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (**1**)

Tetrasaccharide **13** (0.838 g, 0.500 mmol) was dissolved in a mixture of 20% acetic acid in methanol (58 mL). Following the addition of palladium (10 wt. % on activated carbon), the reaction was stirred for 16 h at RT under an atmosphere of hydrogen. The mixture was filtered through Celite and a Millex-FGS (0.2 μm) filter unit. The solution was then concentrated under reduced pressure and co-evaporated with toluene to give a white residue. Reverse phase chromatography on C-18 silica using water: methanol furnished Le^y derivative **1** as a white solid (0.364 g, 94%). $[\alpha]^{22}_D$ = -108.1° (*c* 0.42, H_2O); ^{1}H NMR (D_2O, 600 MHz): δ 5.27 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1'''), 5.08 (d, 1 H, $J_{1,2}$

= 3.9 Hz, H-1'), 4.87 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 6.8$ Hz, H-5'), 4.54 (d, 1 H, $J_{1,2} = 7.8$ Hz, H-1), 4.50 (d, 1 H, $J_{1,2} = 7.9$ Hz, H-1''), 4.25 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 6.7$ Hz, H-5'''), 4.03 (ddd, 1 H, $^2J = 10.2$ Hz, $^3J = 4.9$ Hz, 10.2 Hz, $\mathrm{C\underline{Ha}CH_2Si}$), 4.01 (dd, 1 H, $^2J = 12.1$ Hz, $J_{5,6} = 1.8$ Hz, H-6a), 3.93-3.88 (m, 2 H, H-3', H-4), 3.87-3.62 (m, 14 H, $\mathrm{\underline{CHb}CH_2Si}$, H-2, H-3, H-6b, H-2', H-4', H-2'', H-3'', H-4'', H-6''a, H-6''b, H-2''', H-3''', H-4'''), 3.58 (ddd, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 4.3$ Hz, 7.8 Hz, H-5''), 3.43 (ddd, 1 H, $J_{4,5} = 9.8$ Hz, $J_{5,6} = 2.0$ Hz, 5.5 Hz, H-5), 2.02 (s, 3 H, Ac), 1.27 (d, 3 H, H-6'''), 1.23 (d, 3 H, H-6'), 0.97 (ddd, 1 H, $^2J = 13.8$, $^3J = 7.4$ Hz, 10.5 Hz, $\mathrm{C\underline{Ha}SiMe_3}$), 0.86 (ddd, 1 H, $^3J = 4.8$ Hz, 9.7 Hz, $\mathrm{C\underline{Hb}SiMe_3}$), 0.00 (s, 9 H, $\mathrm{SiMe_3}$); ^{13}C NMR (D_2O, 125 MHz): δ 174.8, 101.1, 101.0, 100.2, 99.4, 77.2, 76.5, 75.9, 75.7, 74.4, 74.2, 72.8, 72.6, 70.6, 70.0, 69.6, 69.3, 69.1, 68.6, 67.7, 67.7, 62.3, 60.8, 56.8, 49.8, 23.3, 18.1, 16.4, -1.4; HRMS (ES) calcd. for $C_{31}H_{57}NO_{19}NaSi$ (M + Na) 798.3192, found *m/z* 798.3192.

2-(Trimethylsilyl)ethyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside (**2**)

Pentasaccharide **17** (0.0572 g, 28 μmol) was dissolved in a mixture of 20% acetic acid in methanol (10 mL). Palladium (10 wt. % on activated carbon) was added and the reaction was stirred at RT under an atmosphere of hydrogen for 16 h. The mixture was filtered through Celite and a Millex-FGS (0.2 μm) filter. Co-evaporation of the resulting solution with toluene gave a white solid. Reverse phase (C-18) HPLC in water: acetonitrile yielded Le^y derivative **2** as a white solid (0.0148 g, 56%). $[\alpha]^{22}_D = -85.4°$ (*c* 0.56, H_2O); ^{1}H NMR (D_2O, 600 MHz): δ 5.28 (d, 1 H, $J_{1,2} = 3.5$ Hz, H-1''''), 5.12 (d, 1 H, $J_{1,2} = 3.8$ Hz, H-1''), 4.88 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 6.8$ Hz, H-5''), ca. 4.76 (overlap with HOD, 1 H, H-1'), 4.52 (d, 1 H, $J_{1,2} = 7.7$ Hz, H-1'''), 4.40 (d, 1 H, $J_{1,2} = 8.1$, H-1), 4.26 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 6.7$ Hz, H-5''''), 4.14 (dd, 1 H, $J_{3,4} = 3.5$ Hz, $J_{4,5} < 1$ Hz, H-4), 4.03 (ddd, 1 H, $^2J = 9.9$ Hz, $^3J = 5.1$ Hz, 12.5 Hz, $\mathrm{C\underline{Ha}CH_2Si}$), 4.01 (dd, 1 H, $^2J = 12.1$ Hz, $J_{5,6}$

= 1.8 Hz, H-6'a), 3.97-3.91 (m, 3 H, H-2', H-4', H-3''), 3.90-3.64 (m, 17 H, CHbCH$_2$Si, H-3, H-5, H-6a, H-6b, H-3', H-6b', H-2'', H-4'', H-2''', H-3''', H-4''', H-6'''a, H-6'''b, H-2'''', H-3'''', H-4''''), 3.60 (dd, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 4.4 Hz, 7.8 Hz, H-5'''), 3.54 (dd, 1 H, $J_{2,3}$ = 9.7 Hz, H-2), 3.46 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6b}$ = 5.0 Hz, H-5'), 2.03 (s, 3 H, Ac), 1.27 (d, 3 H, H-6''''), 1.24 (d, 3 H, H-6''), 1.07 (ddd, 1 H, ^2J = 13.0 Hz, ^3J = 5.3 Hz, 13.0 Hz, CHaSiMe$_3$); 0.98 (ddd, 1 H, ^3J = 5.3 Hz, 12.8 Hz, CHbSiMe$_3$), -0.03 (s, 9 H, SiMe$_3$); ^{13}C NMR (D$_2$O, 125 MHz): δ175.5, 103.1, 102.9, 101.0, 100.2, 99.3, 83.2, 77.1, 76.2, 75.6, 75.5, 75.3, 74.3, 73.8, 72.7, 72.5, 70.5, 69.9, 69.5, 69.1, 69.0, 68.5, 67.7, 67.5, 62.2, 61.5, 60.6, 57.0, 23.0, 18.3, 16.2, 16.2, -0.3; HRMS (ES) calcd. for $C_{37}H_{67}NO_{24}NaSi$ (M + Na) 960.3720, found *m/z* 960.3711; elemental analysis calcd (%) for $C_{37}H_{67}NO_{24}Si\cdot 4H_2O$: C 44.0, H 7.5, N 1.4, found: C 43.9, H 7.2, N 1.3.

2-(Trimethylsilyl)ethyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside (**3**)

A mixture of hexasaccharide **21** (impure, < 33 μmol, based on starting material **20**) and palladium (10 wt. % on activated carbon) in 20% acetic acid in methanol (5 mL) was stirred for 16 h at RT under an atmosphere of hydrogen. Following filtration through Celite and a Millex-FGS (0.2 μm) filter, the solution was co-evaporated with toluene. The residue was subjected to reverse-phase (C-18) HPLC in water: methanol. Subsequent lyophilization gave the hexasaccharide Le^y derivative **3** as a white solid (0.016 g, 44% over 2 steps from **20**). $[\alpha]^{22}_D$ = -82.0° (*c* 0.30, CH$_3$OH); ^{1}H NMR (D$_2$O, 600 MHz): δ 5.23 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1'''''), 5.12 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1'''), 4.87 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 7.0 Hz, H-5'''), ca. 4.81 (overlap with HOD, 1 H, H-1''), 4.51 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1''''), 4.50 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1), 4.44 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1'), 4.25 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.8 Hz, H-5'''''), 4.14 (dd, 1 H, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ < 1 Hz, H-4'), 4.03 (ddd, 1 H, ^2J = 9.9 Hz, ^3J = 5.2 Hz , 12.6 Hz, CHaCH$_2$Si), 4.00 (dd, 1 H, $^2J = 13.2$ Hz , $J_{5,6} = 2.0$ Hz, H-6''a), 3.97 (dd, 1 H, $^2J = 12.3$ Hz, $J_{5,6} = 2.1$ Hz, H-6a), 3.95-3.90 (m, 2 H, H-4'', H-3'''), 3.88-3.55 (m, 24 H, CHbCH$_2$Si, H-3, H-4, H-5, H-6b, H-2', H-3', H-5', H-6'a, H-6'b, H-2'', H-3'', H-6''b, H-2''', H-4''', H-2'''', H-3'''', H-4'''', H-5'''', H-6''''a, H-6''''b, H-2''''', H-3''''', H-4'''''), 3.45 (ddd, 1 H, $J_{4,5} = 9.9$ Hz, $J_{5,6b} = 4.9$ Hz, H-5''), 3.29 (m, 1 H, higher order, H-2), 2.03 (s, 3 H, Ac), 1.27 (d, 3 H, H-6'''''), 1.24 (d, 3 H, H-6'''), 1.07 (ddd, 1 H, $^2J = 13$ Hz , $^3J = 5.5$ Hz, 13 Hz, 1 H, CHaSiMe$_3$), 0.98 (ddd, 1 H, $^3J = 5.1$ Hz, 12.6 Hz, CHbSiMe$_3$), -0.03 (s, 9 H, SiMe$_3$); ^{13}C NMR (D$_2$O, 125 MHz): δ 175.4, 103.7, 103.3, 102.2, 101.0, 100.2, 99.4, 82.8, 79.2, 77.2, 76.2, 75.7, 75.6, 75.4, 74.4, 73.9, 73.7, 72.8, 72.6, 70.9, 70.6, 70.0, 69.6, 69.3, 69.1, 68.6, 67.8, 67.6, 62.3, 61.8, 61.0, 60.7, 57.0, 23.2, 18.5, 16.4, -1.6; HRMS (ES) calcd. for $C_{43}H_{77}NO_{29}NaSi$ (M + Na) 1122.4248, found *m/z* 1122.4264; elemental analysis calcd (%) for $C_{43}H_{77}NO_{29}Si \cdot 2H_2O$: C 45.5, H 7.2, N 1.2, found: C 45.6, H 7.0, N 1.1.

4-Methylphenyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-3-O-acetyl-6-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**5**)

Alcohol **9** (impure, ca. 8 mmol) was treated with dry pyridine (50 mL) and acetic anhydride (25 mL, 0.26 mol). The solution was stirred at RT for 18 h and then concentrated under reduced pressure. Co-evaporation three times with toluene (20 mL) yielded yellow oil. Column chromatography in toluene: ethyl acetate (7:1) gave **5** as a white solid (5.68 g, 69% over two steps from **6**). $[\alpha]^{22}_{D} = +19.5°$ (*c* 1.4, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.85-7.67 (m, 4 H, Phth), 7.34- 6.96 (m, 24 H, Ar), 5.65 (dd, 1 H, $J_{2,3} = 10.2$ Hz, $J_{3,4} = 9.0$ Hz, H-3), 5.61 (d, 1 H, $J_{1,2} = 10.4$ Hz, H-1), 5.17 (dd, 1 H, $J_{1,2} = 7.9$ Hz, $J_{2,3} = 10.1$ Hz, H-2'), 4.87 (d, 1 H, $^2J = 11.7$ Hz, PhCH$_2$O), 4.65 (d, 1 H, $^2J = 12.0$ Hz, PhCH$_2$O), 4.60 (d, 1 H, $^2J = 12.2$ Hz, PhCH$_2$O), 4.48 (d, 1 H, $^2J = 12.0$ Hz, PhCH$_2$O), 4.47 (d, 1 H, $^2J = 11.7$ Hz, PhCH$_2$O), 4.41 (d, 1 H, $^2J = 12.2$ Hz, PhCH$_2$O), 4.39 (d, 1 H, H-1'), 4.36 (d, 1 H, $^2J = 11.7$ Hz, PhCH$_2$O), 4.33 (d, 1 H, $^2J = 11.7$ Hz, PhC$\underline{H}_2$O), 4.19 (dd, 1 H, H-2), 3.93-3.88 (m, 2 H, H-4, H-4'), 3.78-3.73 (m, 2 H, higher order, H-6a, H-6b), 3.65 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 2.9 Hz, 2.9 Hz, H-5), 3.55 (dd, 1 H, ^2J = 8.6 Hz, $J_{5,6}$ = 8.6 Hz, H-6'a), 3.45 (dd, 1 H, $J_{5,6}$ = 4.9 Hz, H-6'b), 3.36 (dd, 1 H, $J_{4,5}$ < 1 Hz, H-5'), 3.33 (dd, 1 H, $J_{3,4}$ = 2.9 Hz, H-3'), 2.25 (s, 3 H, PhC$\underline{H}_3$), 1.92 (s, 3 H, Ac), 1.70 (s, 3 H, Ac); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 169.8, 169.0, 167.6, 167.3, 138.5, 138.3, 138.2, 137.9, 137.7, 134.2, 134.0, 133.7, 131.8, 131.3, 129.6, 128.4, 128.4, 128.3, 128.2, 128.2, 128.1, 127.8, 127.8, 127.8, 127.7, 127.6, 127.4, 127.4, 127.3, 123.6, 128.4, 100.8, 83.1, 80.4, 79.2, 77.4, 77.2, 76.9, 75.2, 74.4, 73.6, 73.5, 73.5, 73.3, 72.5, 72.3, 68.1, 68.0, 54.2, 21.3, 21.1, 20.6; HRMS (ES) calcd. for $C_{59}H_{59}NO_{13}SNa$ (M + Na) 1044.3605, found *m/z* 1044.3613; elemental analysis calcd (%) for $C_{59}H_{59}NO_{13}S$: C 69.3, H 5.8, N 1.4, found C 69.2, H 5.8, N 1.4.

4-Methylphenyl 6-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (6)

A mixture of crushed 3 Å MS (12 g), methyl orange (0.003 mg, 0.009 mmol), and 4-methylphenyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-*β*-D-glucopyranoside (8)[81] (5.719 g, 11.36 mmol) in dry tetrahydrofuran (40 mL) was stirred for 1 h under argon at RT. Sodium cyanoborohydride (4.28 g, 68.11 mmol) was then added. To this stirred mixture, a freshly prepared solution of HCl in ether was added dropwise until the reaction turned pink and the evolution of gas ceased. Upon completion of the reaction (ca. 1 hour), a saturated aqueous solution of sodium bicarbonate was added carefully until the reaction turned orange and the evolution of gas ceased. The mixture was then diluted with dichloromethane and filtered through Celite. The organic phase was washed sequentially with saturated sodium bicarbonate solution, water, and then brine. The organic phase was dried over anhydrous sodium sulphate and then evaporated under reduced pressure. The resultant oil was subjected to column chromatography in hexane:ethyl acetate (2:3) to give a white solid (4.046 g, 70%). $[\alpha]^{22}_D$ = +17.3° (*c* 0.71, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.90-6.98 (m, 13 H, Ar), 5.54 (d, 1 H, $J_{1,2}$ = 10.3

Hz, H-1), 4.60 (d, 1 H, ^2J = 11.7 Hz, PhC$\underline{H}_2$O), 4.56 (d, 1 H, PhC$\underline{H}_2$O), 4.33 (dd, 1 H, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 8.2 Hz, H-3), 4.19 (dd, 1 H, H-2), 3.84 (dd, 1 H, ^2J =10.2 Hz, $J_{5,6}$ = 4.4 Hz, H-6a), 3.77 (dd, 1 H, $J_{5,6}$ = 4.9 Hz, H-6b), 3.68-3.58 (m, 2 H, H-5, H-4), 2.26 (s, 3 H, PhC$\underline{H}_3$); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 168.2, 138.0, 137.7, 134.2, 132.9, 131.5, 129.6, 128.4, 128.3, 127.8, 127.7, 83.8, 78.5, 73.7, 72.7, 72.6, 70.1, 55.8, 21.2; HRMS (ES) calcd. for $C_{28}H_{27}NO_6SNa$ (M + Na) 528.1457, found *m/z* 528.1449.

4-Methylphenyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-6-O-benzyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**9**)

Crushed 4 Å MS (12 g) was added to a round bottom flask containing diol **6** (4.05 g, 8.00 mmol) and trichloroacetimidate **7**[79] (6.63 g, 10.4 mmol). The flask was purged with argon, dry dichloromethane (60 mL) was added, and the mixture was stirred for 2 hours. The mixture was cooled to –25 °C and treated with trimethylsilyl trifluoromethanesulfonate (0.2 mL, 1 mmol). The reaction was stirred for 1 hour at -25°C and then was quenched by addition of excess triethylamine. The mixture was filtered through Celite and the solution was concentrated under reduced pressure. Column chromatography in hexane:ethyl acetate (4:1) rendered a small quantity of pure **9** as a white solid, sufficient for characterization, while the majority of the product was collected along with small amounts of co-eluting, unidentified impurities and used without further attempts at purification. $[\alpha]^{22}_D$ = +17.9° (*c* 1.5, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.88-6.94 (m, 28 H, Ar), 5.49 (d, 1 H, $J_{1,2}$ = 10.6 Hz, H-1), 5.33 (dd, 1 H, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 10.1 Hz, H-2'), 4.86 (d, 1 H, ^2J = 11.7 Hz, PhC$\underline{H}_2$O), 4.62 (d, 2 H, ^2J = 11.9 Hz, 2 PhC$\underline{H}_2$O), 4.48 (m, 2 H, ^2J =11.7 Hz, ^2J = 11.9 Hz, 2 PhC$\underline{H}_2$O), 4.47 (d, 1 H, ^2J = 11.9 Hz, PhC$\underline{H}_2$O), 4.41 (dd, 1 H, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 7.7 Hz, H-3), 4.36 (d, 1 H, H-1'), 4.30 (d, 1 H, ^2J = 11.5 Hz, PhC$\underline{H}_2$O), 4.25 (d, 1 H, ^2J = 11.5 Hz, PhC$\underline{H}_2$O), 4.20 (dd, 1 H, H-2), 3.82 (d, 1 H, $J_{3,4}$ = 2.9 Hz, $J_{4,5}$ <1 Hz, H-4'), 3.72 (dd, 1 H, ^2J = 11.2 Hz, $J_{5,6}$ = 1.5 Hz, H-6a), 3.68 (dd, 1 H, $J_{5,6}$ = 3.7 Hz, H-6b), 3.66-3.58 (m, 2

H, H-5, H-4), 3.56-3.51 (m, 2 H, H-5', H-6'a), 3.43-3.36 (m, 2 H, H-3', H-6'b), 2.24 (s, 3 H, PhC$\underline{H}_3$), 1.96 (s, 3 H, Ac); ^{13}C NMR (CDCl$_3$, 150 MHz): δ 133.8, 133.6, 129.5, 128.3, 128.1, 127.8, 127.5, 127.3, 123.6, 123.1, 101.7, 83.4, 81.5, 80.0, 78.2, 74.3, 73.6, 73.5, 73.4, 72.0, 72.0, 71.1, 70.8, 68.3, 68.3, 55.1, 20.8, 20.7; HRMS (ES) calcd. for $C_{57}H_{57}NO_{12}SNa$ (M + Na) 1002.3499, found *m/z* 1002.3494; elemental analysis calcd. (%) for $C_{57}H_{57}NO_{12}S$: C 69.9, H 5.9, N 1.4, found C 69.8, H 5.9, N 1.5.

2-(Trimethylsilyl)ethyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-3-O-acetyl-6-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**11**)

Bromine (15 μL, 0.291 mmol) was added to a solution of thioglycoside **5** (0.150 g, 0.147 mmol) in dry dichloromethane (5 mL) under argon at 0 °C. The reaction was stirred for 15 minutes, quenched by addition of cyclohexene (0.06 mL, 0.59 mmol), and then transferred via a canula to a round bottom flask containing a stirred suspension of 2-(trimethylsilyl)ethanol **10** (0.03 mL, 0.209 mmol), mercury(II) cyanide (0.056 g, 0.22 mmol), and mercury(II) bromide (0.010 g, 0.028 mmol) in dry dichloromethane (5 mL) at RT. Upon completion of the reaction, the mixture was diluted with ethyl acetate and washed with saturated potassium iodide solution, followed by saturated sodium bicarbonate solution, water, and then brine. The organic solution was dried over sodium sulfate and then concentrated. Chromatography of the residue using hexane:ethyl acetate (5:2) gave **11** as a waxy solid (0.111 g, 74%). $[\alpha]^{22}_{D}$ = +17.8° (*c* 0.45, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.84-7.66 (m, 4 H, Phth), 7.34-7.10 (m, 20 H, Ar); 5.66 (dd, 1 H, $J_{2,3}$ = 10.7 Hz, $J_{3,4}$ = 8.8 Hz, H-3), 5.33 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1), 5.21 (dd, 1 H, $J_{1,2}$ = 8.0 Hz, $J_{2,3}$ = 10.1 Hz, H-2'), 4.89 (d, 1 H, ^{2}J = 11.7 Hz, PhC$\underline{H}_2$O), 4.72 (d, 1 H, ^{2}J =12.1 Hz, PhC$\underline{H}_2$O), 4.62 (d, 1 H, ^{2}J =12.2 Hz, PhC$\underline{H}_2$O), 4.51 (d, 1 H, ^{2}J = 12.1 Hz, PhC$\underline{H}_2$O), 4.50 (d, 1 H, ^{2}J = 11.7 Hz, PhC$\underline{H}_2$O), 4.46-4.34 (m, 4 H, 3 PhC$\underline{H}_2$O, H-1'), 4.17 (dd, 1 H, H-2), 3.98-3.90 (m, 3 H, H-4, C$\underline{Ha}$CH$_2$Si, H-4'), 3.80-3.73 (m, 2 H, H-6a, H-6b), 3.64 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 1.9 Hz, 3.6 Hz, H-5), 3.58 (dd, 1 H, ^{2}J = 8.6 Hz, $J_{5,6}$ = 8.6

Hz, H-6'a), 3.53-3.46 (m, 2 H, $\mathrm{C\underline{Hb}CH_2Si}$, H-6'b), 3.38 (dd, 1 H, $J_{4,5}$ <1 Hz, $J_{5,6}$ = 5.5 Hz, H-5'), 3.34 (dd, 1 H, $J_{3,4}$ = 2.8 Hz, H-3'), 1.94 (s, 3 H, Ac), 1.74 (s, 3 H, Ac), 0.82 (ddd, 1 H, ^2J = 14.0 Hz, ^3J = 6.7 Hz, 10.4 Hz, $\mathrm{C\underline{Ha}SiMe_3}$), 0.73 (ddd, 1 H, ^3J = 5.6 Hz, 9.9 Hz, $\mathrm{C\underline{Hb}SiMe_3}$), 0.15 (s, 9 H, $\mathrm{SiMe_3}$); ^{13}C NMR ($\mathrm{CDCl_3}$, 125 MHz): δ 169.8, 169.0, 138.5, 138.2, 137.9, 137.7, 128.4, 128.3, 128.3, 128.1, 127.8, 127.8, 127.7, 127.6, 127.6, 127.3, 127.3, 100.8, 97.5, 80.4, 75.6, 74.8, 74.4, 73.6, 73.5, 73.2, 72.3, 71.8, 68.0, 67.9, 67.0, 60.4, 60.2, 55.2, 22.3, 21.1, 20.6, 17.9, 14.3, -1.4; HRMS (ES) calcd. for $\mathrm{C_{57}H_{65}NO_{14}SiNa}$ (M + Na) 1038.4072, found *m/z* 1038.4073; elemental analysis calcd (%) for $\mathrm{C_{57}H_{65}NO_{14}Si}$: C 67.4, H 6.5, N 1.4, found: C 67.5, H 6.5, N 1.3.

2-(Trimethylsilyl)ethyl 3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-6-O-benzyl-2-acetamido-2-deoxy-β-D-glucopyranoside **(12)**

A solution of disaccharide **11** (1.431 g, 1.408 mmol) and ethylenediamine (4.7 mL, 70 mmol) in 1-butanol (30 mL) was heated to reflux at 116 °C for 16 h. The solution was concentrated under reduced pressure and then co-evaporated with toluene and ethanol. The resulting oil was dissolved in methanol (50 mL) and to this solution was added acetic anhydride (50 mL). The reaction was stirred for 16 h at RT and then concentrated to dryness. Column chromatography using hexane:acetone (2:1) gave **12** as a waxy solid (0.750 g, 63%). $[\alpha]^{22}_{D}$ = -8.0° (*c* 1.9, $\mathrm{CHCl_3}$); ^{1}H NMR ($\mathrm{CDCl_3}$, 600 MHz): δ 7.35-7.24 (m, 20 H, Ar); 5.55 (d, 1 H, $J_{NH,2}$ = 7.0 Hz, NH), 4.85 (d, 1 H, ^2J = 11.5 Hz, $\mathrm{PhC\underline{H_2}O}$), 4.79 (d, 1 H, $J_{1,2}$ = 8.2 Hz, H-1), 4.68 (d, 1 H, ^2J = 11.9 Hz, $\mathrm{PhC\underline{H_2}O}$), 4.66 (d, 1 H, ^2J = 11.9 Hz, $\mathrm{PhC\underline{H_2}O}$), 4.60-4.52 (m, 3 H, 3 $\mathrm{PhC\underline{H_2}O}$), 4.42 (d, 1 H, ^2J = 11.7 Hz, $\mathrm{PhC\underline{H_2}O}$), 4.37 (d, 1 H, ^2J = 11.7 Hz, $\mathrm{PhC\underline{H_2}O}$), 4.25 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1'), 4.01 (dd, 1 H, $J_{2,3}$ = 9.3 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 3.94 (ddd, 1 H, J = 5.4 Hz, 9.6 Hz, 11.1 Hz, $\mathrm{C\underline{Ha}CH_2Si}$), 3.89 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2'), 3.83-3.74 (m, 3 H, H-4',H-6a, H-6b), 3.60-3.45 (m, 6 H, $\mathrm{C\underline{Hb}CH_2Si}$, H-4, H-5, H-5', H-6'a, H-6'b), 3.35-3.25 (m, 2 H, H-2, H-3'), 1.96 (s, 3 H, Ac), 0.91 (m, 2 H, higher order, $\mathrm{C\underline{H_2}SiMe_3}$), -0.02 (s, 9 H, $\mathrm{SiMe_3}$); ^{13}C NMR ($\mathrm{CDCl_3}$, 125 MHz): 170.5, 138.3, 138.0, 138.0, 137.5, 128.4, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 104.2, 99.6, 82.9, 81.9, 74.6, 73.9, 73.6, 73.5, 73.4, 72.9, 72.7, 71.8, 71.1, 69.7, 68.4, 66.9, 57.5, 23.7, 18.2, -1.2; HRMS (ES) calcd. for $C_{47}H_{61}NO_{11}SiNa$ (M + Na) 866.3912, found *m/z* 866.3906; elemental analysis calcd (%) for $C_{47}H_{61}NO_{11}Si$: C 66.9, H 7.3, N 1.7, found: C 66.7, H 7.5, N 1.6.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-benzyl-2-deoxy-β-D-glucopyranoside (**13**)

Bromine (0.4 mL, 8 mmol) was added dropwise to a stirred solution of ethyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside[83] (2.94 g, 6.14 mmol) in dry dichloromethane (15 mL) at 0 °C under argon. After complete conversion of the starting material to bromide **4**[82] (ca. 20 minutes), the reaction was quenched by addition of cyclohexene (1.6 mL, 16 mmol). This solution was then transferred under argon via canula to a round bottom flask containing a stirred suspension of diol **12** (0.649 g, 0.769 mmol), tetraethylammonium bromide (0.355 g, 1.69 mmol), crushed 4 Å MS (5.5 g), dry DMF (10 mL) and dry dichloromethane (10 mL). The reaction was stirred for 2 days under argon at RT. Excess triethylamine was added to quench the reaction and the mixture was stirred for an additional 3 hours before it was filtered through Celite and the solution was concentrated. Sequential chromatography on two columns using first hexane:acetone (3:1) and then toluene:acetone (9:1) gave tetrasaccharide **13** as a white solid (0.906 g, 70%). $[\alpha]^{22}_D$ = -59.9° (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.36-7.04 (m, 50 H, Ar), 5.65 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1'''), 5.56 (d, 1 H, $J_{NH,2}$ = 6.5 Hz, NH), 5.16 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1), 4.89 (dd, 2 H, ^2J = 11.1 Hz, PhC$\underline{H_2}$O), 4.86 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1'), 4.78-4.47 (m, 16 H, 14 PhC$\underline{H_2}$O, H-5', H-1''), 4.42 (dd, 2 H, ^2J = 11.9 Hz, PhC$\underline{H_2}$O), 4.35 (d, 1 H, ^2J = 10.7 Hz, PhC$\underline{H_2}$O), 4.28 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5'''), 4.22 (dd, 1 H, $J_{2,3}$ = 9.4 Hz, $J_{3,4}$ = 9.4 Hz, H-3), 4.09-4.00 (m, 3 H, H-2', H-2'', H-2'''), 3.98- 3.90 (m, 4

H, C$\underline{\mathrm{Ha}}$CH$_2$Si, PhC$\underline{\mathrm{H_2}}$O, H-4, H-4''), 3.85 (dd, 1 H, $J_{2,3}$ = 10.2 Hz, $J_{3,4}$ = 2.5 Hz, H-3'), 3.81-3.60 (m, 6 H, H-6a, H-6b, H-6''a, H-6''b, H-3''', H-4'''), 3.57-3.51 (m, 2 H, C$\underline{\mathrm{Hb}}$CH$_2$Si, H-3''), 3.33 (ddd, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6}$ = 5.1 Hz, 8.7 Hz, H-5''), 3.25 (d, 1 H, $J_{4,5}$ = 9.4 Hz, $J_{5,6} < 1$ Hz, H-5), 3.19 (d, 1 H, $J_{4,5} < 1$ Hz, H-4'), 2.91 (dd, 1 H, H-2), 1.54 (s, 3 H, NAc), 1.28 (d, 3 H, H-6'''), 1.10 (d, 3 H, $J_{5,6}$ = 6.5 Hz, H-6'), 0.95 (ddd, 1 H, ^2J = 13.8 Hz, ^3J = 6.5 Hz, 10.6 Hz, C$\underline{\mathrm{Ha}}$SiMe$_3$), 0.86 (ddd, 1 H, ^3J = 5.4 Hz, 10.2 Hz, C$\underline{\mathrm{Hb}}$SiMe$_3$), 0.00 (s, 9 H, SiMe$_3$); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 170.2, 139.2, 139.0, 138.8, 138.8, 138.7, 138.7, 138.5, 138.4, 138.0, 137.7, 128.7, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.6, 127.6, 127.5, 127.4, 127.3, 127.2, 127.2, 127.1, 127.1, 127.0, 126.1, 100.1, 98.4, 98.2, 97.8, 84.0, 80.3, 79.1, 78.4, 78.3, 76.2, 75.6, 75.6, 75.5, 74.9, 74.3, 73.9, 73.5, 73.3, 73.3, 73.1, 72.5, 72.4, 72.4, 71.8, 71.1, 68.3, 68.0, 67.0, 66.9, 66.7, 60.4, 23.4, 18.1, 16.4, 16.4, -1.2; MS (ES) calcd. for $C_{101}H_{117}NO_{19}SiNa$ (M + Na) 1698.8, found *m/z* 1698.8, correct isotopic intensity pattern.

2-(Trimethylsilyl)ethyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-3-O-acetyl-6-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→ 3)-2-O-benzyl-4,6-O-benzylidene-β-D-galactopyranoside (**15**)

A mixture of thioglycoside donor **5** (2.00g, 1.96 mmol), alcohol **14**[76] (1.10 g, 2.41 mmol) and crushed 4 Å MS (10 g) in dry dichloromethane (30 mL) was stirred at RT under argon for 2 h and then was cooled to –30 °C. The round bottom flask was covered in aluminum foil, *N*-iodosuccinmide (0.661 g, 2.94 mmol) and silver trifluoromethanesulfonate (0.352g, 1.37 mmol) were added to the mixture, and the reaction was stirred for 2 h at –30 °C. The reaction was quenched by addition of excess triethylamine. It was then diluted with dichloromethane and filtered through Celite. The organic solution was washed sequentially with saturated aqueous sodium thiosulfate, aqueous sodium bicarbonate, water, and then brine. Drying of the organic solution over sodium sulphate was followed by evaporation of the solvent under reduced pressure. The residue was subjected to sequential column chromatography on two columns using first toluene:acetone (8:1) and then hexane:ethyl acetate (3:2). This yielded a few fractions of pure **15**, but most of the product (2.46 g, ca. 92% yield) was collected along with a small quantity of unidentified, co-eluting impurities and was used without further attempts at purification. $[\alpha]^{22}_D = +6.0°$ (*c* 4.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): *δ* 7.60-6.93 (m, 34 H, Ar), 5.70-5.64 (m, 2 H, H-1', H-3'), 5.41 (s, 1 H, $PhC\underline{H}O_2$), 5.22 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.1 Hz, H-2''), 4.88 (d, 1 H, ^2J = 11.5 Hz, $PhC\underline{H}_2O$), 4.61 (d, 1 H, ^2J = 12.1 Hz, $PhC\underline{H}_2O$), 4.60 (d, 1 H, ^2J = 11.5 Hz, $PhC\underline{H}_2O$), 4.50-4.48 (m, 2 H, 2 $PhC\underline{H}_2O$), 4.44-4.28 (m, 6 H, 4 $PhC\underline{H}_2O$, H-1'', H-2'), 4.26-4.23 (m, 3 H, H-4, H-1, $PhC\underline{H}_2O$), 4.18 (dd, 1 H, ^2J = 11.9 Hz, $J_{5,6}$ = 1.3 Hz, H-6a), 3.91 (d, 1 H, $J_{3,4}$ = 3.91 Hz, $J_{4,5}$ < 1 Hz, H-4''), 3.91-3.84 (m, 2 H, $C\underline{Ha}CH_2Si$, H-4'), 3.77 (dd, 1 H, $J_{5,6}$ = 1.6 Hz, H-6b), 3.74-3.68 (m, 3 H, H-5', H-6'a, H-6'b), 3.66 (dd, 1 H, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 3.6 Hz, H-3), 3.60-3.55 (m, 2 H, H-2, H-6''a), 3.45 (dd, 1 H, ^2J = 8.8 Hz, $J_{5,6}$ = 4.8 Hz, H-6''b), 3.40-3.34 (m, 3 H, H-5'', H-3'', $C\underline{Hb}CH_2Si$), 3.24 (s, 1 H, H-5), 1.92 (s, 3 H, OAc), 1.72 (s, 3 H, OAc), 0.81 (m, 2 H, higher order, $C\underline{H}_2SiMe_3$), -0.11 (s, 9 H, $SiMe_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 169.7, 169.0, 138.8, 138.4, 138.0, 138.0, 137.8, 137.6, 128.5, 128.4, 128.3, 128.3, 128.1, 127.9, 127.8, 127.8, 127.8, 127.7, 127.6, 127.6, 127.6, 127.4, 127.3, 126.8, 126.8, 126.7, 126.2, 103.1, 100.9, 100.7, 99.0, 80.9, 80.3, 77.2, 75.9, 75.6, 74.5, 74.4, 74.2, 73.7, 73.5, 73.3, 72.1, 72.0, 71.8, 71.7, 69.0, 67.9, 67.3, 66.3, 55.3, 21.1, 20.6, 18.3, -1.4; HRMS (ES) calcd. for $C_{77}H_{85}NO_{19}SiNa$ (M + Na) 1378.5383, found *m/z* 1378.5378; elemental analysis calcd (%) for $C_{77}H_{85}NO_{19}Si$: C 68.2, H 6.3, N 1.0, found: C 68.2, H 6.4, N 1.0.

2-(Trimethylsilyl)ethyl 3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-2-acetamido-6-O-benzyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2-O-benzyl-4,6-O-benzylidene-β-D-galactopyranoside (**16**)

A solution of phthalimido derivative **15** (1.68 g, 1.24 mmol) and ethylenediamine (4.2

mL, 63 mmol) in 1-butanol (38 mL) was heated to reflux at 116 °C for 16 h. The solution was concentrated under reduced pressure and then co-evaporated with toluene and ethanol. The resulting oil was dissolved in methanol (40 mL) and treated with acetic anhydride (20 mL, 212 mmol). The reaction was stirred for 16 h at room temperature and then concentrated to dryness. Column chromatography using hexane:acetone (3:2) gave **16** as a glassy solid (1.22 g, 83%). $[\alpha]^{22}_{D}$ = +10.3° (*c* 0.71, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 7.50-7.23 (m, 30 H, Ar), 5.40 (s, 1 H, $PhC\underline{H}O_2$), 5.25 (d, 1 H, $J_{NH,2}$ = 6.4 Hz, NH), 5.03 (d, 1 H, ^2J = 12.0 Hz, $PhC\underline{H}_2O$), 4.88-4.83 (m, 2 H, H-1', $PhC\underline{H}_2O$), 4.67 (dd, 2 H, higher order, $PhC\underline{H}_2O$), 4.59 (d, 1 H, ^2J = 12.0 Hz, $PhC\underline{H}_2O$), 4.56-4.51 (m, 2 H, 2 $PhC\underline{H}_2O$), 4.46 (d, 1 H, ^2J = 11.9 Hz, $PhC\underline{H}_2O$), 4.39 (d, 1 H, ^2J = 11.9 Hz, $PhC\underline{H}_2O$), 4.35 (d, 1 H, $J_{1,2}$ = 7.6 Hz, H-1), 4.34 (d, 1 H, ^2J = 11.9 Hz, $PhC\underline{H}_2O$), 4.28 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1''), 4.25-4.21 (m, 2 H, H-4, H-5), 4.00 (ddd, 1 H, J = 6.4 Hz, 9.8 Hz, 10.7 Hz, $C\underline{Ha}CH_2Si$), 3.93 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2''), 3.88 (dd, 1 H, ^2J = 11.0 Hz, $J_{5,6}$ = 2.44 Hz, H-6'a), 3.87-3.80 (m, 3 H, H-6a, H-3', H-4''), 3.76 (dd, 1 H, $J_{2,3}$ = 9.6 Hz, H-2), 3.70-3.64 (m, 2 H, H-3, H-6'b), 3.60-3.40 (m, 7 H, H-5', H-5'', $C\underline{Hb}CH_2Si$, H-6''a, H-4', H-6b'', H-2'), 3.34 (dd, 1 H, $J_{3,4}$ = 2.8 Hz, H-3''), 3.25 (s, 1 H, ^2J < 1 Hz, $J_{5,6}$ < 1 Hz, H-6b), 1.47 (s, 3 H, NAc), 0.97 (m, 2 H, higher order, $C\underline{H_2}SiMe_3$), -0.02 (s, 9 H, $SiMe_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.4, 139.2, 138.4, 138.1, 137.9, 137.6, 129.7, 128.9, 128.6, 128.5, 128.4, 128.4, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.6, 127.5, 127.5, 127.3, 126.8, 126.4, 103.7, 103.2, 101.5, 101.0, 81.8, 80.4, 80.1, 78.1, 75.9, 74.6, 74.3, 74.0, 73.9, 73.6, 73.5, 73.3, 73.0, 72.7, 72.6, 70.5, 70.2, 69.0, 68.5, 67.3, 66.4, 60.4, 57.6, 23.0, 18.5, -1.3; HRMS (ES) calcd. For $C_{67}H_{81}NO_{16}SiNa$ (M + Na) 1206.5222, found *m/z* 1206.5217; elemental analysis calcd (%) for $C_{67}H_{81}NO_{16}Si$: C 67.9, H 6.9, N 1.2, found C 67.7, H 6.8, N 1.2.

*2-(Trimethylsilyl)ethyl 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-*

6-O-benzyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2-O-benzyl-4,6-O-benzylidene-β-D-galactopyranoside (**17**)

Bromine (25 μL, 0.48 mmol) was added dropwise to a stirred solution of ethyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside[83] (0.188 g, 0.393 mmol) in dry dichloromethane (2 mL) at 0° C. When complete conversion of the thioglycoside to bromide **4**[82] was observed by TLC (ca. 20 minutes), cyclohexene (50 μL, 0.49 mmol) was added and the solution was transferred under argon via canula to a round bottom flask containing a stirred mixture of crushed 4 Å MS (0.35 g), diol **16** (0.058 g, 49 μmol), tetraethylammonium bromide (0.023 g, 0.11 mmol), dry DMF (1.5 mL) and dry dichloromethane (1.5 mL). The reaction was stirred for 5 days at RT under argon. Excess triethylamine was added and the mixture was stirred for an additional 3 h. The mixture was diluted with dichloromethane, filtered through Celite, and concentrated to a yellow oil before being subjected to column chromatography using hexane:ethyl acetate (3:1). The reaction yielded 0.052 g (52%) of pentasaccharide **17** as a white solid. $[\alpha]^{22}_D$ = -37.1° (*c* 0.97, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.52-6.98 (m, 60 H, Ar), 5.65 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1’’’’), 5.44 (d, 1 H, $J_{NH,2}$ = 6.6 Hz, NH), 5.42 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1’), 5.38 (s, 1 H, PhC$\underline{H}O_2$), 4.90 (d, 1 H, ^{2}J = 11.5 Hz, PhC$\underline{H}_2$O), 4.82 (d, 1 H, ^{2}J = 12.3 Hz, PhC$\underline{H}_2$O), 4.82 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1’’), 4.80 (d, 1 H, ^{2}J = 11.4 Hz, PhC$\underline{H}_2$O), 4.74 (d, 1 H, ^{2}J = 12.1 Hz, PhC$\underline{H}_2$O), 4.72 (d, 1 H, ^{2}J = 10.6 Hz, PhC$\underline{H}_2$O), 4.69 (d, 1 H, ^{2}J = 12.1 Hz, PhC$\underline{H}_2$O), 4.64-4.50 (m, 11 H, 6 PhC$\underline{H}_2$O, H-5’’, 3 PhC$\underline{H}_2$O, H-1’’’), 4.47 (d, 1 H, ^{2}J = 12.1 Hz, PhC$\underline{H}_2$O), 4.44 (d, 1 H, ^{2}J = 11.7 Hz, PhC$\underline{H}_2$O), 4.41 (d, 1 H, ^{2}J = 11.9 Hz, PhC$\underline{H}_2$O), 4.39-4.33 (m, 3 H, 3 PhC$\underline{H}_2$O), 4.33-4.28 (m, 3 H, H-3’, H-1, H-5’’’’), 4.25-4.20 (m, 2 H, H-4, H-6a), 4.08-4.04 (m, 2 H, H-2’’’’, H-2’’’), 4.01-3.87 (m, 5 H, C$\underline{Ha}$CH$_2$Si, H-4’’’, H-2’’, PhC$\underline{H}_2$O, H-4’), 3.83 (dd, 1 H, $J_{2,3}$ = 10.2 Hz, $J_{3,4}$ = 2.6 Hz, H-3’’), 3.81-3.77 (m, 3 H, H-6’a, H-3’’’’, H-6b), 3.73-3.68 (m, 3 H, H-2, H-6’’’a, H-4’’’’), 3.65-3.56 (m, 4 H, H-3, H-6’b, H-6’’’b, H-3’’’), 3.50 (ddd, 1 H, J = 5.7 Hz, 9.5 Hz, 11.5 Hz, C$\underline{Hb}$CH$_2$Si), 3.41-3.37 (m, 2 H, H-5’, H-5’’’), 3.26 (s, 1 H, $J_{4,5}$, $J_{5,6}$

<1 Hz, H-5), 3.16 (d, 1 H, $J_{4,5}$ < 1 Hz, H-4''), 3.05 (ddd, 1 H, $J_{2,3}$ = 9.3 Hz, H-2'), 1.29 (d, 3 H, $J_{5,6}$ = 6.4 Hz, H-6''''), 1.20 (s, 3 H, Ac), 1.09 (d, 3 H, $J_{5,6}$ = 6.4 Hz, H-6''), 0.94 (m, 2 H, higher order, $C\underline{H}_2SiMe_3$), -0.03 (s, 9 H, $SiMe_3$); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 128.3, 128.0, 127.4, 127.0, 126.8, 126.4, 126.0, 103.0, 100.8, 100.0, 100.0, 97.9, 97.8, 83.7, 80.9, 80.0, 79.2, 78.1, 78.0, 77.2, 75.4, 75.4, 75.3, 75.4, 75.2, 74.5, 74.5, 74.5, 73.4, 73.3, 73.3, 73.3, 73.2, 73.2, 72.8, 72.3, 72.2, 72.1, 71.4, 70.7, 68.9, 68.8, 67.7, 67.1, 66.6, 66.3, 66.0, 60.3, 22.6, 18.2, 15.9, 15.9, -0.1; MS (ES) calcd. for $C_{121}H_{137}NO_{24}SiNa$ (M + Na) 2038.9, found *m/z* 2040.0, correct isotopic intensity pattern.

2-(Trimethylsilyl)ethyl 2-O-acetyl-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-3-O-acetyl-6-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**19**)

A mixture of lactose derivative **18**[87] (0.316g, 0.321 mmol), thioglycoside donor **5** (0.394 g, 0.385 mmol), and crushed 4 Å MS (2.5 g) were stirred in dry dichloromethane (5 mL) at RT under argon for 2 h. The mixture was cooled to –30 °C and treated with *N*-iodosuccinimide (0.087 g, 0.39 mmol) and silver trifluoromethanesulfonate (0.066 g, 0.26 mmol). The round bottom flask was covered with aluminum foil and the reaction was stirred for 2 h between –30 and –10 °C. The mixture was diluted with dichloromethane, filtered through Celite, and the solution was washed sequentially with saturated aqueous sodium thiosulfate, saturated aqueous sodium bicarbonate, water, and then brine. The organic phase was dried over sodium sulphate and then concentrated under reduced pressure. Column chromatography on the residue using hexane:ethyl acetate (3:1) gave **19** (0.47 g, 77 %) as a glassy solid. $[\alpha]^{22}_D$ = +0.1° (*c* 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 600 MHz): δ 7.70-6.86 (m, 54 H, Ar), 5.73 (dd, 1 H, $J_{2,3}$ = 10.6 Hz, $J_{3,4}$ = 8.8 Hz, H-3''), 5.57 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1''), 5.19 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.1 Hz, H-2'''), 5.00 (d, 1 H, ^2J = 11.4 Hz, $PhC\underline{H}_2O$), 4.87 (d, 1 H, ^2J = 11.7 Hz, $PhC\underline{H}_2O$), 4.85 (d, 1 H, ^2J = 10.5 Hz, $PhC\underline{H}_2O$), 4.81 (d, 1 H, ^2J = 11.1 Hz, $PhC\underline{H}_2O$), 4.65 (d, 1 H, ^2J = 11.1 Hz, $PhC\underline{H}_2O$), 4.60 (m, 2 H, ^2J = 12.3 Hz, ^2J = 11.7 Hz, 2 PhC$\underline{H}_2$O), 4.53 (d, 1 H, ^2J = 10.5 Hz, PhC$\underline{H}_2$O), 4.48 (d, 1 H, ^2J = 11.7 Hz, PhC$\underline{H}_2$O), 4.46 (d, 1 H, ^2J = 11.4 Hz, PhC$\underline{H}_2$O), 4.44-4.37 (m, 4 H, 2 PhC$\underline{H}_2$O, H-1’’’, PhC$\underline{H}_2$O), 4.35 (d, 1 H, ^2J = 11.5 Hz, PhC$\underline{H}_2$O), 4.33 (d, 1 H, ^2J = 11.5 Hz, PhC$\underline{H}_2$O), 4.30 (d, 1 H, ^2J = 11.9 Hz, PhC$\underline{H}_2$O), 4.28-4.21 (m, 4 H, PhC$\underline{H}_2$O, H-1’, H-2”, PhC$\underline{H}_2$O), 4.19 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1), 4.15 (d, 1 H, ^2J = 11.9 Hz, PhC$\underline{H}_2$O), 4.05 (d, 1 H, ^2J = 11.9 Hz, PhC$\underline{H}_2$O), 3.99 (d, 1 H, $J_{3,4}$ = 3.1 Hz, $J_{4,5}$ <1 Hz, H-4’), 3.96 (dd, 1 H, $J_{4,5}$ = 9.8 Hz, H-4”), 3.92-3.86 (m, 2 H, H-4’’’, C$\underline{Ha}$CH$_2$Si), 3.82-3.74 (m, 2 H, H-4, H-6”a), 3.71 (dd, 1 H, ^2J = 10.8 Hz, $J_{5,6}$ = 1.7 Hz, H-6”b), 3.66 (ddd, 1 H, $J_{5,6}$ = 3.5 Hz, H-5”), 3.58-3.53 (m, 2 H, H-3’, H-6’’’a), 3.50-3.42 (m, 6 H, C$\underline{Hb}$CH$_2$Si, H-6’’’b, H-6a, H-2’, H-6’a, H-6’b), 3.38-3.30 (m, 5 H, H-5’’’, H-5’, H-3’’’, H-3, H-6b), 3.26 (dd, 1 H, $J_{2,3}$ = 9.2 Hz, H-2), 2.94 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 1.8 Hz, 4.0 Hz, H-5), 1.91 (s, 3 H, Ac), 1.70 (s, 3 H, Ac), 0.94 (m, 2 H, C$\underline{H}_2$SiMe$_3$), -0.04 (s, 9 H, SiMe$_3$); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 169.6, 168.9, 139.3, 139.0, 138.8, 138.5, 138.4, 138.3, 138.2, 138.0, 137.9, 137.6, 128.4, 128.3. 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.6, 127.6, 127.6, 127.5, 127.4, 127.3, 127.2, 127.1, 126.9, 126.7, 126.4, 103.0, 102.3, 100.8, 99.3, 83.0, 82.2, 81.8, 80.4, 78.7, 76.0, 75.4, 75.3, 75.1, 74.9, 74.7, 74.4, 74.4, 74.0, 73.7, 73.5, 73.3, 73.2, 73.0, 72.2, 71.8, 71.7, 71.3, 68.2, 67.9, 67.8, 67.2, 55.6, 21.1, 20.6, 18.5, -1.3; MS (ES) calcd. for $C_{111}H_{121}NO_{24}SiNa$ (M + Na) 1902.8, found *m/z* 1903.8.

*2-(Trimethylsilyl)ethyl 3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→ 4)-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→ 4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside* (**20**)

The phthalimido derivative **19** (0.934 g, 0.496 mmol) was treated with ethylenediamine (1.66 mL, 24.8 mmol) in 1-butanol (80 mL). The solution was heated to reflux at 116 °C for 16 h, concentrated under reduced pressure, and co-evaporated with toluene and ethanol. The residue was dissolved in methanol (15 mL) and acetic anhydride (15 mL, 159 mmol). The reaction was stirred for 16 h at room temperature and then concentrated to dryness. Column chromatography using toluene:ethyl acetate (2:1) gave amide **20** (0.635 g, 75%) as a white solid. $[\alpha]^{22}_{D}$ = -5.7° (*c* 1.5, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.36-7.10 (m, 50 H, Ar), 4.98 (d, 1 H, ^{2}J = 10.6 Hz, PhC$\underline{H}_2$O), 4.97 (d, 1 H, ^{2}J = 11.7 Hz, PhC$\underline{H}_2$O), 4.92-4.84 (m, 4 H, PhC$\underline{H}_2$O, NH, 2 PhC$\underline{H}_2$O), 4.74-4.64 (m, 5 H, 3 PhC$\underline{H}_2$O, H-1'', PhC$\underline{H}_2$O), 4.59 (d, 1 H, ^{2}J = 12.8 Hz, PhC$\underline{H}_2$O), 4.56-4.48 (m, 5 H, 5 PhC$\underline{H}_2$O), 4.44-4.30 (m, 6 H, PhC$\underline{H}_2$O, H-1', 2 PhC$\underline{H}_2$O, H-1, PhC$\underline{H}_2$O), 4.28 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1'''), 4.18 (d, 1 H, ^{2}J = 11.9 Hz, PhC$\underline{H}_2$O), 3.98-3.88 (m, 4 H, C$\underline{Ha}$CH$_2$Si, H-4', H-2''', H-4), 3.87-3.83 (m, 2 H, H-6a'', H-4'''), 3.78-3.62 (m, 5 H, H-6''b, H-2'', H-6a, H-2', H-6b), 3.59-3.48 (m, 10 H, H-3', H-5''', H-6'''a, H-6'''b, H-4'', H-5'', H-3'', C$\underline{Hb}$CH$_2$Si, H-3, H-6'a), 3.41 (dd, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.4 Hz, 6.4 Hz, H-5'), 3.38-3.33 (m, 2 H, H-3''', H-2), 3.28 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 1.8 Hz, 4.22 Hz, H-5), 1.52 (s, 3 H, NAc), 0.99 (m, 2 H, higher order, C$\underline{H_2}$SiMe$_3$), -0.01 (s, 9 H, SiMe$_3$); ^{13}C NMR (CDCl$_3$, 150 MHz): δ 128.1, 127.9, 127.7, 127.0, 125.8, 103.9, 102.9, 102.4, 102.0, 82.7, 81.7, 81.4, 80.0, 76.5, 76.1, 75.2, 74.8, 74.7, 74.6, 74.5, 74.2, 74.2, 73.5, 73.3, 73.2, 73.2, 73.1, 73.0, 72.6, 72.4, 70.5, 69.4, 68.1, 68.1, 68.0, 67.1, 66.9, 56.0, 22.8, 18.2, 0.02; MS (ES) calcd. for C$_{101}$H$_{117}$NO$_{21}$SiNa (M + Na) 1730.8, found *m/z* 1731.8, correct isotopic intensity pattern.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-benzyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-benzyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-benzyl-β-D-galactopyranosy-(1→ 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**21**)

Bromine (0.25 mL, 4.9 mmol) was added dropwise to a stirred solution of ethyl 2,3,4-tri-*O*-benzyl-1-thio-*β*-L-fucopyranoside[83] (1.97 g, 4.12 mmol) in dry dichloromethane (3 mL) at 0° C. When complete conversion of the thioglycoside to bromide **4**[82] was observed (ca. 20 minutes), cyclohexene (1.1 mL, 11 mmol) was added and the solution was transferred under argon via canula to a round bottom flask containing a stirred mixture of crushed 4 Å MS (2.5 g), diol **20** (0.585 g, 0.342 mmol), and tetraethylammonium bromide (0.158 g, 0.752 mmol) in dry DMF (1.5 mL) and dry dichloromethane (2.5 mL). The reaction was stirred for 3 days at room temperature under argon. Excess triethylamine was added and the reaction was stirred for an additional 3 h. The mixture was diluted, filtered through Celite, and concentrated to a yellow oil before being subjected to sequential column chromatography on two columns using first toluene:ethyl acetate (6:1) and then hexane:ethyl acetate (3:1). No further attempt at purification was carried out and 0.73 g of hexasaccharide **21** (with ca. 5% impurities) was obtained. ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.40-7.00 (m, 80 H, Ar), 5.64 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1’’’’’), 5.31-5.26 (m, 2 H, NH, H-1’’), 5.05 (d, 1 H, ^2J = 11.5 Hz, PhC$\underline{H}_2$O), 4.95 (d, 1 H, ^2J = 10.6 Hz, PhC$\underline{H}_2$O), 4.90 (d, 1 H, ^2J = 11.4 Hz, PhC$\underline{H}_2$O), 4.85 (d, 1 H, ^2J = 11.2 Hz, PhC$\underline{H}_2$O), 4.79 (d, 1 H, ^2J = 4.0 Hz, H-1’’’), 4.76 (d, 1 H, ^2J = 12.3 Hz, PhC$\underline{H}_2$O), 4.75-4.45 (m, 21 H, 20 PhC$\underline{H}_2$O, H-1’’’’), 4.42-4.40 (m, 2 H, PhC$\underline{H}_2$O, H-1’), 4.40-4.30 (m, 3 H, 3 PhC$\underline{H}_2$O), 4.30-4.20 (m, 4 H, PhC$\underline{H}_2$O, H-1, H-5’’’’’, H-3’’), 4.17 (d, 1 H, ^2J = 11.7 Hz, PhC$\underline{H}_2$O), 4.10 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 7.1 Hz, H-5’’’), 4.06-4.00 (m, 4 H, H-2’’’’’, H-4’’, H-4’, H-2’’’’), 3.97-3.90 (m, 3 H, C$\underline{\text{Ha}}$CH$_2$Si, H-4’’’’, H-2’’’), 3.90-3.85 (m, 2 H, PhC$\underline{H}_2$O, H-4), 3.82-3.78 (m, 2 H, H-6’’a, H-3’’’), 3.75 (dd, 1 H, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.8 Hz, H-3’’’’’), 3.71-3.62 (m, 4 H, H-6’’’’a, H-4’’’’’, H-2’, H-6a), 3.60 (dd, 1 H, ^2J = 10.6 Hz, $J_{5,6}$ = 1.6 Hz, H-6b), 3.58-3.39 (m, 8 H, H-6’’’’b, H-3’, C$\underline{\text{Hb}}$CH$_2$Si, H-3’’’’, H-6’’b, H-6’a, H-3, H-5’), 3.37- 3.30 (m, 3 H, H-6’b, H-2, H-5’’’’), 3.27 (ddd, 1 H, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 1.8 Hz, 2.6 Hz, H-5’’), 3.18 (ddd, 1 H, $J_{4,5}$ = 9.5 Hz, $J_{5,6}$ = 4.4 Hz, H-5), 3.14 (d, 1 H, $J_{3,4}$ = 1.1 Hz, H-4’’’), 3.08 (m, 1 H, H-2’’), 1.26-1.24 (m, 9 H, H-6’’’’’, NAc, H-6’’’), 0.98 (m, 2 H, higher order, C$\underline{H}_2$SiMe$_3$), -0.01 (s, 9 H, SiMe$_3$); MS (ES) calcd. for $C_{155}H_{173}NO_{29}SiNa$ (M + Na) 2563.2, found *m/z* 2564.2, correct isotopic intensity pattern.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-β-D-galactopyranoside (**33**)

Pentasaccharide **2** (0.0604 g, 64.4 μmol) was treated with dry pyridine (10 mL) and acetic anhydride (5 mL, 53 mmol). The solution was stirred at RT for 2 days and then concentrated under reduced pressure. The residue was co-evaporated three times and then subjected to column chromatography in toluene:acetone (5:2), after which **33** was obtained as a white solid (0.079 g, 83%). $[\alpha]^{22}_{D}$ = -91.3° (*c* 2.4, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.41 (d, 1 H, $J_{NH,2}$ = 7.1 Hz, NH), 5.36 (d, 1 H, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ < 1 Hz, H-4), 5.33-5.29 (m, 4 H, H-4’’, H-4’’’, H-1’’’’, H-4’’’’), 5.26 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1’’), 5.15 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.3 Hz, H-3’’), 5.11-5.08 (m, 2 H, H-1’, H-3’’’’), 5.05 (dd, 1 H, $J_{1,2}$ = 8.1 Hz, $J_{2,3}$ = 10.1 Hz, H-2), 5.02 (dd, 1 H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.7 Hz, H-3’’’), 5.00-4.91 (m, 4 H, H-6’a, H-2’’, H-5’’, H-2’’’’), 4.58 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1’’’), 4.47 (dd, 1 H, ^2J = 11.4 Hz, $J_{5,6}$ = 5.7 Hz, H-6’’’a), 4.44-4.38 (m, 3 H, H-1, H-3’, H-5’’’’), 4.26 (dd, 1 H, $J_{5,6}$ = 8.3 Hz, H-6’’’b), 4.13 (dd, 1 H, ^2J = 11.4 Hz, $J_{5,6}$ = 6.8 Hz, H-6a), 4.08-4.03 (m, 2 H, H-6b, H-6’b), 3.95 (ddd, 1 H, ^2J = 9.9 Hz, ^3J = 5.1 Hz, 10.6 Hz, C$\underline{\text{Ha}}$CH$_2$Si), 3.88-3.78 (m, 4 H, H-3, H-5, H-4’, H-5’’’), 3.76 (dd, 1 H, H-2’’’), 3.54 (ddd, 1 H, ^3J = 9.9 Hz, 6.6 Hz, C$\underline{\text{Hb}}$CH$_2$Si), 3.42 (ddd, 1 H, $J_{4,5}$ = 10.0 Hz, $J_{5,6}$ = 2.9 Hz, H-5’), 2.86 (ddd, 1 H, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 7.5 Hz, H-2’), 2.16 (s, 3 H, OAc), 2.13 (s, 3 H, OAc), 2.12 (s, 3 H, OAc), 2.12 (s, 3 H, OAc), 2.10 (s, 3 H, OAc), 2.10 (s, 3 H, OAc), 2.07 (s, 6 H, 2 OAc), 2.04 (s, 3 H, OAc), 1.98 (s, 3 H, OAc), 1.97 (s, 3 H, OAc), 1.96 (s, 3 H, OAc), 1.95 (s, 3 H, OAc), 1.90 (s, 3 H, NAc), 1.19 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’’), 1.16 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’), 0.96 (ddd, 1 H, ^2J = 13.9 Hz, ^3J = 10.8 Hz, 6.6 Hz, C$\underline{\text{Ha}}$SiMe$_3$), 0.88 (ddd, 1 H, ^3J = 10.4 Hz, 5.3 Hz, C$\underline{\text{Hb}}$SiMe$_3$), -0.02 (s, 9 H, SiMe$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.2, 170.7, 170.6, 170.5, 170.5, 170.4, 170.2, 170.2, 170.1, 170.0, 169.8, 169.8,169.6, 169.6, 100.7, 100.4, 99.1, 96.2, 95.6, 77.3, 77.0, 76.7, 76.6, 74.1, 73.3. 72.9, 72.6, 72.6, 71.5, 71.1, 71.0, 70.9, 70.8, 69.9, 68.7, 67.9, 67.7, 67.5, 67.5, 66.9, 65.1, 64.0, 61.8, 60.6, 60.4, 59.8, 31.0, 23.6, 21.2, 21.2, 21.1, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.7, 18.0, 16.0, 15.6, -1.3; MS (ES) calcd. for $C_{63}H_{93}NO_{37}SiNa$ (M + Na) 1506.5, found *m/z* 1506.4, correct isotopic intensity pattern.

2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-α-D-galactopyranosyl trichloroacetimidate (**34**)

Trifluoroacetic acid (1.5 mL, 20 mmol) was added to a solution of 2-(trimethylsilyl)ethyl glycoside **33** (0.0311 g, 21.0 μmol) in dry dichloromethane (0.75 mL) at 0 °C under argon. The reaction was stirred for 30 minutes, the ice bath was removed, and then stirring was continued for an additional 30 minutes. Ethyl acetate (ca. 1 mL) and toluene (ca. 2 mL) were added to the reaction. The solution was then concentrated under reduced pressure and co-evaporated twice with toluene. The resulting residue was dissolved in dry dichloromethane (3.5 mL) and the solution was cooled to –5 °C. The solution was treated with trichloroacetonitrile (0.1 mL, 1 mmol) and then the dropwise addition of DBU (4 μL, 27 μmol). The mixture was stirred for 2 h under argon at –5 °C, during which the solution turned brown. The solution was evaporated under reduced pressure and the resulting residue was subjected to column chromatography using toluene:acetone (5:2) to give trichloroacetimidate **34** as a glassy solid (0.0261 g, 81%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.63 (s, 1 H, C=N$\underline{\text{H}}$), 6.50 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.54 (d, 1 H, $J_{3,4}$ = 3.5 Hz, $J_{4,5}$ < 1 Hz, H-4), 5.36 (d, 1 H, $J_{NH,2}$ = 7.7 Hz, NH), 5.35-5.26 (m, 6 H, H-1’’’’, H-4’’, H-4’’’, H-1’’, H-4’’’’, H-2), 5.16 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.3 Hz, H-3’’), 5.13 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.1 Hz , H-3’’’’), 5.04-4.95 (m, 4 H, H-1’, H-3’’’, H-2’’’’, H-2’’), 4.91 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5’’), 4.79 (dd, 1 H, ^{2}J = 12.1 Hz, $J_{5,6}$ = 2.0 Hz, H-6’a), 4.53 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1’’’), 4.50 (dd, 1 H, ^{2}J = 11.4 Hz, $J_{5,6}$

= 5.7 Hz, H-6’’’a), 4.40 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6} = 6.6$ Hz, H-5’’’’), 4.36 (dd, 1 H, $J_{5,6} =$ 6.4 Hz, 6.4 Hz, H-5), 4.29 (dd, 1 H, $J_{2,3} = 9.3$ Hz, $J_{3,4} = 9.3$ Hz, H-3’), 4.26 (dd, 1 H, $J_{5,6} =$ 8.4 Hz, H-6’’’b), 4.17-4.10 (m, 3 H, H-3, H-6’b, H-6a), 4.01 (dd, 1 H, $^2J = 11.5$ Hz, H-6b), 3.87 (dd, 1 H, $J_{4,5} = 9.5$ Hz, H-4’), 3.84 (dd, 1 H, $J_{4,5} < 1$ Hz, H-5’’’), 3.75 (dd, 1 H, $J_{2,3} = 9.9$ Hz, H-2’’’), 3.52 (ddd, 1 H, $J_{5,6} = 4.6$ Hz, H-5’), 3.09 (ddd, 1 H, $J_{1,2} = 7.7$ Hz, H-2’), 2.16 (s, 3 H, Ac), 2.13 (s, 6 H, 2 Ac), 2.12 (s, 6 H, 2 Ac), 2.11 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.02 (s, 6 H, 2 Ac), 1.99 (s, 3 H, Ac), 1.97 (s, 3 H, Ac), 1.96 (s, 3 H, Ac), 1.95 (s, 3 H, Ac), 1.88 (s, 3 H, Ac), 1.19 (d, 3 H, H-6’’’’), 1.16 (d, 3 H, H-6’’); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.9, 170.6, 170.5, 170.5, 170.4, 170.3, 170.2, 170.0, 169.7, 169.6, 169.3, 161.0, 100.5, 99.5, 96.3, 95.6, 93.7, 74.1, 73.3, 73.3, 73.0, 73.0, 72.7, 71.4, 71.1, 70.9, 69.9, 69.7, 68.6, 68.5, 67.9, 67.8, 67.4, 66.9, 65.2, 64.1, 62.0, 61.2, 60.5, 23.5, 21.2, 21.0, 20.9, 20.9, 20.8, 20.8, 20.7, 16.0, 15.8; MS (ES) calcd. for $C_{60}H_{81}Cl_3N_2O_{37}Na$ (M + Na) 1549.3, found *m/z* 1549.3, correct isotopic intensity pattern.

*(2S,3R)-2-azido-3-benzoyloxy-4-pentenyl 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranoside* **(35)**

A mixture of trichloroacetimidate **34** (0.0285 g, 18.6 μmol), (2*S*,3*R*)-2-azido-3-benzoyloxy-4-penten-1-ol (0.0294g, 0.119 mmol), and crushed 4 Å MS (1.2 g) in dry dichloromethane (1.0 mL) and trimethylacetonitrile (2.0 mL) was stirred for 3 h under argon. The mixture was cooled to 0 °C and boron trifluoride diethyl etherate (39 μL, 0.31 mmol) was added dropwise. The ice bath was removed and the reaction was stirred at RT for 18 h. The mixture was cooled again to 0 °C and a second aliquot of boron trifluoride diethyl etherate (9 μL, 70 μmol) was added. The reaction was again allowed to warm to room temperature and stirred for 24 h. Excess triethylamine was added at 0 °C and the mixture was stirred for a 2 h. The mixture was then diluted with dichloromethane and filtered through Celite. Concentration of the filtered solution gave a residue that was subjected to column chromatography in toluene:acetone (5:2) to give alkene **35** (0.0206g, 68%) as a solid. $[\alpha]^{22}_{D}$ = -72.9° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.06-7.42 (m, 5 H, Ar), 5.92 (ddd, 1 H, ^3J = 7.1 Hz, 10.4 Hz, 17.4 Hz, $CH_2{=}C\underline{H}$), 5.64 (dd, 1 H, ^3J = 3.7 Hz, 7.1 Hz, $C\underline{H}OBz$), 5.48-5.30 (m, 8 H, $C{=}C\underline{H_2}$, NH, H-4, H-1'''', H-4'', H-4''', H-4''''), 5.27 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1''), 5.16 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.3 Hz, H-3''), 5.12-5.08 (m, 3 H, H-3'''', H-2, H-1'), 5.03 (dd, 1 H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 3.7 Hz, H-3'''), 5.01-4.91 (m, 4 H, H-6'a, H-2'''', H-2'', H-5''), 4.59 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1'''), 4.48 (dd, 1 H, ^2J = 11.5 Hz, $J_{5,6}$ = 5.7 Hz, H-6'''a), 4.45-4.37 (m, 3 H, H-1, H-5'''', H-3'), 4.26 (dd, 1 H, $J_{5,6}$ = 8.4 Hz, H-6'''b), 4.10-4.02 (m, 3 H, H-6a, H-6'b, H-6b), 3.98-3.93 (m, 2 H, CHN_3, $OC\underline{Ha}HbCHN_3$), 3.89-3.80 (m, 4 H, H-4', H-5''', H-3, H-5), 3.77 (dd, 1 H, H-2'''), 3.59 (m, 1 H, higher order, $OCHaC\underline{Hb}CHN_3$), 3.42 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 2.9 Hz, 2.9 Hz, H-5'), 2.88 (ddd, 1 H, $J_{1,2}$ = 7.7 Hz, $J_{2,3}$ = 7.7 Hz, $J_{NH,2}$ = 7.7 Hz, H-2'), 2.16 (s, 3 H, Ac), 2.14 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.12 (s, 3 H, Ac), 2.11 (s, 6 H, 2 Ac), 2.05 (s, 6 H, 2 Ac), 1.99 (s, 3 H, Ac), 1.97 (s, 6 H, 2 Ac), 1.96 (s, 3 H, Ac), 1.91 (s, 3 H, Ac), 1.20 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6''''), 1.16 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6''); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.1, 170.7, 170.6, 170.5, 170.4, 170.4, 170.2, 170.2, 170.1, 170.0, 169.8, 169.8, 169.6, 169.5, 164.9, 133.2, 131.2, 129.7, 129.6, 128.4, 120.7, 101.0, 100.4, 99.1, 96.2, 95.7, 76.3, 74.7, 74.0, 73.3, 73.0, 72.6, 72.5, 71.5, 71.4, 70.9, 70.9, 70.3, 69.7, 68.8, 67.9, 67.7, 67.6, 66.9, 65.1, 64.0, 63.3, 61.8, 60.5, 60.2, 29.8, 23.6, 21.2, 21.1, 21.0, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.7, 16.0, 15.6; MS (ES) calcd. for $C_{70}H_{92}N_4O_{39}Na$ (M + Na) 1635.5, found *m/z* 1635.5, correct isotopic intensity pattern.

(2S,3R)-2-azido-3-hydroxy-4-pentenyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside **(36)**

Sodium metal (ca. 10 mg, 0.44 mmol) was added to a solution of *O*-acylated glycoside **35** (0.0285 g, 17.7 μmol) in dry methanol (25 mL) and the solution was stirred for 16 h. The reaction was monitored by TLC using a mixture of acetic acid: water: methanol: chloroform (1:2:5:4) as eluent. The solution was neutralized with Dowex cation exchange resin (H^+ ionic form), filtered, concentrated under reduced pressure, and co-evaporated twice with toluene to quantitatively afford glycoside **36** as a white solid. $[\alpha]^{22}_D$ = -70.6° (*c* 0.33, CH_3OH); ^{1}H NMR (D_2O, 600 MHz): δ 5.93 (ddd, 1 H, ^3J = 6.8 Hz, 10.4 Hz, 17.2 Hz, $CH_2{=}C\underline{H}$), 5.39 (d, 1 H, ^3J = 17.2 Hz, $C\underline{Ha}Hb{=}CH$), 5.35 (d, 1 H, ^3J = 10.4 Hz, $CHa\underline{Hb}{=}CH$), 5.28 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1''''), 5.12 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1''), 4.88 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5''), ca 4.75 (overlap with HOD, 1 H, H-1'), 4.52 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1'''), 4.42 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 4.36 (m, 1 H, higher order, $C{=}CHC\underline{H}OH$), 4.26 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5''''), 4.15 (dd, 1 H, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ < 1 Hz, H-4), 4.01 (dd, 1 H, ^2J = 11.9 Hz, $J_{5,6}$ = 1.6 Hz, H-6'a), 3.97-3.59 (m, 24 H, $OC\underline{H_2}CHN_3$, $OCH_2C\underline{H}N_3$, H-2, H-3, H-5, H-6a, H-6b, H-2', H-3', H-4', H-6'b, H-2'', H-3'', H-4'', H-2''', H-3''', H-4''', H-5''', H-6'''a, H-6'''b, H-2'''', H-3'''', H-4''''), 3.46 (ddd, 1 H, $J_{4,5}$ = 9.2 Hz, $J_{5,6}$ = 1.5 Hz, 4.2 Hz, H-5'), 2.03 (s, 3 H, Ac), 1.27 (d, 3 H, H-6''''), 1.24 (d, 3 H, H-6''); ^{13}C NMR (D_2O, 125 MHz): δ 175.5, 135.9, 119.4, 103.6, 103.2, 101.0, 100.2, 99.4, 83.0, 77.2, 76.2, 75.7, 75.6, 74.4, 73.9, 73.0, 72.8, 72.6, 70.6, 70.5, 70.0, 69.6, 69.3, 69.2, 69.1, 68.6, 67.8, 67.6, 65.7, 62.6, 62.3, 62.3, 61.7, 60.7, 57.1, 23.2, 16.4, 16.4; HRMS (ES) calcd. for $C_{37}H_{62}N_4O_{25}Na$ (M + Na) 985.3601, found *m/z* 985.3608.

(2S,3R)-2-butyramido-3-hydroxy-4-pentenyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside **(37)**

Hydrogen sulfide was bubbled through a stirred solution of azide **36** (0.033 g, 34 μmol) in a mixture of pyridine (55 mL), water (5.5 mL), and triethylamine (2.75 mL) at 0 °C for two days. The reaction was monitored by TLC using a mixture of acetic acid: water: methanol: chloroform (1:2:5:4) as eluent ($Rf_{36} = 0.6$; product $Rf_{37} = 0.2$). The mixture was concentrated under reduced pressure and co-evaporated with toluene. The residue was dissolved in dry methanol (13 mL), cooled to 0 °C, and butyric anhydride (0.2 mL, 1.2 mmol) was added. The ice bath was removed and the reaction stirred for 18 h. Methanolic sodium methoxide (1 M) was added and the reaction was stirred for a further 6 h before the solution was concentrated under reduced pressure. Attempts to purify the residue by reverse phase (C-18) flash chromatography in water:methanol and reverse phase HPLC in water:acetonitrile were unsuccessful. Ceramide analog **37** was collected along with non-carbohydrate impurities from which it could not be separated (0.018 g, in 84% purity as estimated from the NMR spectrum, ca. 44% yield). ^{1}H NMR (D_2O, 600 MHz): *δ* 5.85 (m, 1 H, $H_2C{=}C\underline{H}$), 5.36-5.26 (m, 3 H, $C{=}CH_2$, H-1''''), 5.12 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1''), 4.87 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6}$ = 6.6 Hz, H-5''), 4.72 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1'), 4.51 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1'''), 4.37 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 4.25 (dq, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6}$ = 6.6 Hz, H-5''''), 4.18 (dd, 1 H, ^3J = 6.9 Hz, 6.9 Hz, $C{=}CHC\underline{H}OH$), 4.13 (dd, 1 H, $J_{3,4}$ = 3.1 Hz, $J_{4,5} < 1$ Hz, H-4), 4.02-3.63 (m, 23 H, H-6'a, CHN_3, H-2', H-4' ,H-3'', H-3', H-3''', H-4''', H-6'b, H-4'', H-2'''', H-3'''', H-4'''', H-6a, $OC\underline{Ha}HbCHNH$, H-6'''a, H-6'''b, H-6b, H-5, H-2'', H-3, $OCHa\underline{Hb}CHNH$, H-2'''), 3.60 (ddd, 1 H, $J_{4,5} < 1$ Hz, $J_{5,6}$ = 4.5 Hz, 7.0 Hz, H-5'''), 3.56 (dd, 1 H, $J_{2,3}$ = 8.2 Hz, H-2), 3.45 (ddd, 1 H, $J_{4,5}$ = 7.2 Hz, $J_{5,6}$ = 1.4 Hz, 4.6 Hz, H-5'), 2.24 (ddd, 2 H, ^2J = 7.5 Hz, ^3J = 7.5Hz, 7.5 Hz, $C(O)C\underline{H_2}CH_2CH_3$), 2.03 (s, 3 H, Ac), 1.59 (m, 2 H, $CH_2C\underline{H_2}CH_3$), 1.27 (d, 3 H, H-6''''), 1.24 (d, 3 H, H-6''), 0.90 (dd, 3 H, ^3J = 7.3 Hz, 7.3 Hz, $CH_2CH_2C\underline{H_3}$); ^{13}C NMR (D_2O, 150 MHz): *δ* 137.3, 118.9, 104.1, 103.3, 101.0, 100.2, 99.4, 83.0, 77.2, 76.2, 75.7, 75.5, 74.5, 73.9, 72.9, 72.7, 70.7, 70.6, 69.9, 69.5, 69.1, 69.0, 68.6, 67.7, 67.6, 62.5, 61.4, 60.6, 55.7, 38.6, 23.0, 19.7, 16.3, 13.6; HRMS (ES) calcd. for $C_{41}H_{70}N_2O_{26}Na$ (M + Na) 1029.4109, found *m/z* 1029.4110.

(2S,3R)-8-amino-2-butyramido-3-hydroxy-6-thia-octyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside, acetate salt **(38)**

2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, 0.0375 g, 0.138 mmol) was added to a stirred solution of alkene **37** (ca. 84%, 0.0113 g, ca. 9.4 μmol) and cysteamine hydrochloride (0.0660 g, 0.581 mmol) in degassed water (1.3 mL). The reaction was stirred at 60 °C under argon for 3 days. The reaction progress was monitored by TLC (Rf_{37} = 0.4; Rf_{38} = 0.05) with the eluent system dichloromethane:methanol:water (45:45:10). Cyclohexene (0.3 mL, 3 mmol) was added to the reaction and the solution was concentrated under reduced pressure. The residue was co-evaporated three times with toluene and subjected to size-exclusion chromatography (Bio-Gel P4) and then to reverse phase (C-8) HPLC using 0.3% acetic acid in water:methanol to give amine **38** (acetate salt) as a white solid although small quantities of impurities could not be removed (0.0041 g, ca. 83% purity as estimated from NMR data, ca. 33 % yield). ^{1}H NMR (D_2O, 600 MHz): δ 5.27 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1''''), 5.11 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1''), 4.86 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5''), ca. 4.71 (d, 1 H, $J_{1,2}$ = 8.2 Hz, H-1'), 4.50 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1'''), 4.37 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 4.24 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5''''), 4.18-4.12 (m, 2 H, H-*A*a, H-4), 4.05-3.54 (m, 24 H, H-2, H-3, H-5, H-6a, H-6b, H-2', H-3', H-4', H-6'a, H-6'b, H-2'', H-3'', H-4'', H-2''', H-3''', H-4''', H-5''', H-6'''a, H-6'''b, H-2'''', H-3'''', H-4'''', H-*A*b, H-*B*, H-*C*), 3.47-3.43 (m, 1 H, H-5'), 3.22 (t, 2 H, ^{3}J = 6.7 Hz, H-*G*a, H-*G*b), 2.84 (ddd, 2 H, ^{2}J = 3.1 Hz, H-*F*a, H-*F*b), 2.75 (ddd, 1 H, ^{2}J = 13.1 Hz, ^{3}J = 4.5 Hz, 8.6 Hz, H-*E*a), 2.64 (ddd, 1 H, ^{3}J = 8.2 Hz, 8.2 Hz, H-*E*b), 2.25 (t, 2 H, ^{3}J = 7.3 Hz, H-*H*), 2.02 (s, 3 H, Ac), 1.87-1.79 (m, 1 H, H-*D*a), 1.74-1.68 (m, 1 H, H-*D*b), 1.61 (tq, 2 H, ^{3}J = 7.3 Hz, H-*I*), 1.26 (d, 3 H, H-6''''), 1.23 (d, 3 H, H-6''), 0.91 (t, 3 H, H-*J*); selected ^{13}C HMQC NMR data (D_2O, 150 MHz): δ 104.1, 103.4, 101.0, 100.2, 99.4, 38.4, 23.0, 19.6, 16.1, 13.4; HRMS (ES) calcd. for $C_{43}H_{77}N_3O_{26}SNa$ (M + Na)

1106.4414, found *m/z* 1106.4407.

(2S,3R)-2-butyramido-3-hydroxy-8-(β-maleimidopropionamido)-6-thia-octyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside (**39**)

A 0.180 M solution of 3-maleimidopropionic acid *N*-hydroxysuccinimide ester in DMF (dried over 4 Å MS and pumped for 2 h) was prepared just before use and 60 μL of this solution was added to a solution of amine **38** (0.0041 g, 3.6 μmol) in PBS (0.62 mL, 0.1 M sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 7.2). The reaction was stirred for 4 h at RT, diluted to 10 mL with water, and passed through a pre-washed C-18 Sep-Pak cartridge and Millex filter unit (0.45 μm). The cartridge was washed with water (10 mL) and the product was eluted with methanol (20 mL). Evaporation of the solvent under reduced pressure and lyophilization from water gave maleimide **39** as a white solid (0.0036 g, 81%). ^{1}H NMR (D_2O, 600 MHz): δ 6.86 (s, 2 H, maleimide), 5.27 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1''''), 5.12 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1''), 4.87 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5''), ca 4.73 (overlap with HOD, 1 H, H-1'), 4.51 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1'''), 4.37 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 4.25 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5''''), 4.18-4.12 (m, 2 H, H-*A*a, H-4), 4.06-4.02 (m, 1 H, H-*B*), 3.99 (dd, 1 H, ^2J = 10.7 Hz, $J_{5,6}$ < 1 Hz, H-6'a), 3.96-3.53 (m, 25 H, H-2, H-3, H-5, H-6a, H-6b, H-2', H-3', H-4', H-6'b, H-2'', H-3'', H-4'', H-2''', H-3''', H-4''', H-5''', H-6'''a, H-6'''b, H-2'''', H-3'''', H-4'''', H-*A*b, H-*C*, H-*L*a, H-*L*b), 3.53-3.31 (m, 3 H, H-5', H-*G*), 2.88-2.51 (m, 6 H, H-*E*a, H-*E*b, H-*F*, H-*K*), 2.28-2.24 (m, 2 H, H-*H*), 2.02 (s, 3 H, Ac), 1.86-1.76 (m, 1 H, H-*D*a), 1.72-1.65 (m, 1 H, H-*D*b), 1.62 (tq, 2 H, ^3J = 7.3 Hz, 7.3 Hz, H-*I*), 1.26 (d, 3 H, H-6''''), 1.23 (d, 3 H, H-6''), 0.91 (t, 3 H, H-*J*); MS (ES) calcd. for $C_{50}H_{82}N_4O_{29}SNa$ (M + Na) 1257.5, found *m/z* 1257.

2-(Trimethylsilyl)ethyl 2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-

D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-β-D-galacto-pyranosyl-(1→ 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside **(40)**

2-(Trimethylsilyl)ethyl glycoside **3** (0.1157 g, 0.1052 mmol) was treated with acetic anhydride (7 mL, 74 mmol) and pyridine (13 mL, 161 mmol). The reaction was stirred for 48 h at RT and then concentrated to dryness under reduced pressure. The residue was co-evaporated three times with toluene and subjected to column chromatography in toluene:acetone (5:2) to furnish per-O-acetylated glycoside **40** as a white solid (0.1265 g, 68%). $[\alpha]^{22}_{D}$ = -76.8° (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.39 (d, 1 H, $J_{NH,2}$ = 7.1 Hz, NH), 5.34-5.29 (m, 5 H, H-4’, H-4’’’, H-4’’’’, H-1’’’’’, H-4’’’’’), 5.24 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1’’’), 5.17-5.12 (m, 2 H, H-3, H-3’’’), 5.10 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.2 Hz, H-3’’’’’), 5.07 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1’’), 5.04-4.90 (m, 6 H, H-2’, H-6’’a, H-2’’’, H-5’’’, H-3’’’’, H-2’’’’’), 4.84 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.5 Hz, H-2), 4.59 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1’’’’), 4.51-4.46 (m, 2 H, H-6a, H-6’’’’a), 4.44 (d, 1 H, H-1), 4.42-4.38 (m, 2 H, H-1’, H-5’’’’’), 4.36 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$= 9.5 Hz, H-3’’), 4.25 (dd, 1 H, ^{2}J = 11.4 Hz, $J_{5,6}$ = 8.4 Hz, H-6’’’’b), 4.09-3.98 (m, 4 H, H-6b, H-6’a, H-6’’b, H-6’b), 3.90 (ddd, 1 H, ^{2}J = 9.7 Hz, ^{3}J = 5.7 Hz, 9.7 Hz, $C\underline{Ha}CH_2Si$), 3.86-3.82 (m, 2 H, H-4’’, H-5’’’’), 3.81-3.74 (m, 4 H, H-4, H-3’, H-5’, H-2’’’’), 3.56 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 2.2 Hz, 5.3 Hz, H-5), 3.52 (ddd, 1 H, ^{3}J = 6.6 Hz, 9.7 Hz, $C\underline{Hb}CH_2Si$), 3.40 (ddd, 1 H, $J_{4,5}$ = 10.0 Hz , $J_{5,6}$ = 2.6 Hz, 2.6 Hz, H-5’’), 2.84 (ddd, 1 H, H-2’’), 2.15 (s, 3 H, OAc), 2.13 (s, 6 H, 2 OAc), 2.12 (s, 3 H, OAc), 2.10 (s, 6 H, 2 OAc), 2.09 (s, 3 H, OAc), 2.08 (s, 3 H, OAc), 2.07 (s, 3 H, OAc), 2.04 (s, 3 H, OAc), 2.00 (s, 3 H, OAc), 2.00 (s, 3 H, OAc), 1.98 (s, 3 H, OAc), 1.96 (s, 6 H, 2 OAc), 1.95 (s, 3 H, OAc), 1.90 (s, 3 H, NAc), 1.19 (d, 3 H, $J_{5,6}$ = 6.4 Hz, H-6’’’’’), 1.15 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’), 0.92 (ddd, 1 H, ^{2}J = 13.9 Hz, ^{3}J = 6.6 Hz, 10.3 Hz, CHaSi), 0.85 (ddd, 1 H, ^{3}J = 5.8 Hz, 10.1 Hz, CHbSi), -0.03 (s, 9 H, $SiMe_3$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.2, 170.7, 170.6, 170.5, 170.5, 170.4, 170.2, 170.2, 170.2, 170.1, 170.0, 169.8, 169.7, 169.6, 169.4, 169.4, 169.3, 100.8, 100.4, 99.9, 99.0, 96.2, 95.7, 76.3, 75.8, 74.0, 73.3, 73.1, 73.0, 73.0, 72.6, 72.6, 72.6, 72.6, 72.6, 71.8, 71.4, 71.2, 71.0, 71.0, 70.8, 69.4, 68.7, 67.9, 67.8, 67.5, 67.4, 66.9, 65.2, 64.0, 62.0, 61.4, 60.5, 60.1, 31.0, 23.6, 21.1, 21.0, 20.9, 20.9, 20.9, 20.8, 20.8, 20.8, 20.8, 20.8, 20.7, 20.7, 20.6, 18.0, 16.0, 15.6, -1.3; MS (ES) calcd. for $C_{75}H_{109}NO_{45}SiNa$ (M + Na) 1794.6, found *m/z* 1794.7.

*2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→ 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate* (**41**)

Trifluoroacetic acid (0.9 mL) was added to a stirred solution of per-*O*-acetylated glycoside **40** (0.0266 g, 15.0 μmol) in dry dichloromethane (0.45 mL) at 0 °C under argon. The reaction was stirred for 30 minutes at 0 °C, the ice bath was removed, and the reaction stirred for another 30 minutes. Ethyl acetate (3 mL) and toluene (3 mL) were added and the reaction mixture was concentrated under reduced pressure. The residue was co-evaporated twice with toluene, dissolved in dry dichloromethane (2.5 mL), and the resulting solution was cooled to 0 °C. Trichloroacetonitrile (30 μL, 0.30 mmol) and then DBU (1.1 μL, 7.4 μmol) were added dropwise with stirring. The reaction was stirred at 0 °C under argon for 2 h, during which time the solution turned brown. The mixture was concentrated to dryness under reduced pressure. Flash chromatography on the residue using toluene:acetone:triethylamine (600:200:1) afforded trichloroacetimidate **41** as a white solid (0.0213 g, 78%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.62 (s, 1 H, C=NH), 6.47 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1), 5.49 (dd, 1 H, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H-3), 5.39 (d, 1 H, $J_{NH,2}$ = 7.1 Hz, N$\underline{\text{H}}$Ac), 5.35-5.28 (m, 5 H, H-4’, H-1’’’’’, H-4’’’, H-4’’’’, H-4’’’’’), 5.22 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1’’’), 5.14 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.3 Hz, H-3’’’), 5.08 (dd, 1 H, $J_{2,3}$ = 11.2 Hz, $J_{3,4}$ = 3.2 Hz, H-3’’’’’), 5.06 (d, 1 H, $J_{1,2}$ = 8.2 Hz, H-1’’), 5.05-4.90 (m, 7 H, H-6’’a, H-2, H-3’’’’, H-2’’’’’, H-2’, H-2’’’, H-5’’’), 4.58 (d, 1

H, $J_{1,2}$ = 8.0 Hz, H-1’’’’), 4.54-4.46 (m, 2 H, H-6a, H-6’’’’a), 4.44-4.37 (m, 2 H, H-1’, H-5’’’’’), 4.35 (dd, 1 H, $J_{2,3}$ = 9.8 Hz, $J_{3,4}$ = 9.8 Hz, H-3’’), 4.24 (dd, 1 H, ^2J = 11.5 Hz, $J_{5,6}$ = 8.6 Hz, H-6’’’’b), 4.12-3.8 (m, 5 H, H-6’a, H-5, H-6b, H-6’’b, H-6’b), 3.90-3.70 (m, 6 H, H-4’’, H-5’’’’, H-4, H-5’, H-3’, H-2’’’’), 3.39 (ddd, 1 H, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 2.5 Hz, 2.5 Hz, H-5’’), 2.85 (ddd, 1 H, H-2’’), 2.15 (s, 3 H, Ac), 2.13 (s, 9 H, 3 Ac), 2.12 (s, 3 H, Ac), 2.11 (s, 3 H, Ac), 2.10 (s, 3 H, Ac), 2.10 (s, 3 H, Ac), 2.09 (s, 3 H, Ac), 2.07 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.03 (s, 3 H, Ac), 1.98 (s, 3 H, Ac), 1.96 (s, 6 H, 2 Ac), 1.95 (s, 3 H, Ac), 1.91 (s, 3 H, Ac), 1.19 (d, 3 H, $J_{5,6}$ = 7.0 Hz, H-6’’’’’), 1.16 (d, 3 H, $J_{5,6}$ = 6.5 Hz, H-6’’’); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.2, 170.7, 170.6, 170.5, 170.4, 170.3, 170.2, 170.1, 170.0, 170.0, 169.9, 169.9, 169.6, 169.4, 169.3, 169.3, 160.9, 100.9, 100.4, 99.0, 96.2, 95.7. 92.9, 75.2, 73.9, 73.3, 73.0, 72.6, 71.4, 71.2, 71.1, 71.0, 70.9, 70.8, 70.0, 69.6, 69.4, 68.7, 67.9, 67.7, 67.5, 66.9, 65.2, 64.0, 61.4, 60.5, 60.0, 45.9, 29.8, 23.6, 21.2, 21.0, 21.0, 20.9, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.7, 20.5, 16.0, 15.6; MS (ES) calcd. for $C_{72}H_{97}Cl_3N_2O_{45}Na$ (M + Na) 1837.4, found *m/z* 1837.4, correct isotopic intensity pattern.

(2S,3R)-2-azido-3-benzoyloxy-4-pentenyl 2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside **(42)**

A mixture of trichloroacetimidate **41** (0.0297 g, 16.3 μmol), (2*S*,3*R*)-2-azido-3-benzyloxy-4-penten-1-ol (0.0761 g, 0.308 mmol), crushed 4 Å MS (1.35 g), trimethylacetonitrile (2.6 mL), and dry dichloromethane (1.3 mL) was stirred under argon for 2 h. The reaction was cooled to 0 °C, and boron trifluoride diethyl etherate (39 μL, 0.308 mmol) was added dropwise with stirring. The ice bath was removed and the reaction was stirred for 3 days at RT under argon. The reaction was quenched by addition of excess triethylamine and then was filtered through Celite. The solution was concentrated under reduced pressure and subjected to column chromatography in toluene:acetone (2:1). Glycoside **42** (0.0144 g, 46%) was obtained as a white solid. $[\alpha]^{22}_D$ = -54.5° (*c* 0.40, $CHCl_3$); ^1H NMR ($CDCl_3$, 600 MHz): δ 8.06-7.42 (m, 5 H, Ar), 5.91 (ddd, 1 H, ^3J = 7.0 Hz, 10.4 Hz, 17.4 Hz, $CH_2{=}C\underline{H}$), 5.63 (dd, 1 H, ^3J = 4.0 Hz, 7.1 Hz, $C\underline{H}OBz$), 5.47-5.28 (m, 8 H, $C{=}C\underline{H_2}$, NH, H-4’, H-1’’’’’, H-4’’’, H-4’’’’, H-4’’’’’), 5.25 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1’’’), 5.18-5.14 (m, 2 H, H-3, H-3’’’), 5.12-5.06 (m, 2 H, H-3’’’’’, H-1’’), 5.04-4.88 (m, 7 H, H-6’’a, H-3’’’’, H-2’’’’’, H-2’, H-2’’’, H-5’’’, H-2), 4.60 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1’’’’), 4.53-4.46 (m, 3 H, H-6a, H-1, H-6’’’’a), 4.43-4.38 (m, 2 H, H-5’’’’’, H-1’), 4.36 (dd, 1 H, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$ = 9.7 Hz, H-3’’), 4.26 (dd, 1 H, ^2J = 11.4 Hz, $J_{5,6}$ = 8.6 Hz, H-6’’’’b), 4.09-3.98 (m, 4 H, H-6’a, H-6b, H-6’’b, H-6’b), 3.95 (ddd, 1 H, ^3J = 4.0 Hz, 6.4 Hz, 6.4 Hz, CHN_3), 3.91-3.82 (m, 3 H, $OC\underline{Ha}HbCHN_3$, H-4’’, H-5’’’’), 3.81-3.74 (m, 4 H, H-4, H-3’, H-5’, H-2’’’’), 3.61-3.57 (m, 2 H, H-5, $OCHa\underline{Hb}CHN_3$), 3.41 (ddd, 1 H, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 2.6 Hz, 2.6 Hz, H-5’’), 2.85 (ddd, 1 H, broad, H-2’’), 2.15 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.12 (s, 3 H, Ac), 2.11 (s, 3 H, Ac), 2.10 (s, 3 H, Ac), 2.09 (s, 3 H, Ac), 2.06 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.03 (s, 3 H, Ac), 2.02 (s, 3 H, Ac), 1.99 (s, 3 H, Ac), 1.97 (s, 6 H, 2 Ac), 1.95 (s, 3 H, Ac), 1.90 (s, 3 H, Ac), 1.19 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’’’), 1.16 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.7, 170.7, 170.7, 170.6, 170.5, 170.5, 170.4, 170.2, 170.2, 170.1, 170.0, 169.8, 169.7, 169.6, 169.4, 169.4, 169.3, 164.9, 133.2, 131.2, 129.7, 129.6, 128.4, 120.7, 100.8, 100.4, 100.3, 98.9, 96.2, 95.7, 75.5, 74.6, 73.9, 73.3, 73.0, 72.9, 72.8, 72.6, 71.4, 71.4, 71.2, 71.0, 70.9, 69.4, 68.7, 68.0, 67.9, 67.7, 67.5, 65.2, 64.0, 63.2, 61.7, 61.4, 60.5, 60.1, 23.6, 21.2, 21.0, 20.9, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.7, 16.0, 15.7; MS (ES) calcd. for $C_{82}H_{108}N_4O_{47}Na$ (M + Na) 1923.6, found *m/z* 1923.6.

(2S,3R)-2-azido-3-hydroxy-4-pentenyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-

D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside (**43**)

Sodium metal (ca. 4 mg, 0.2 mmol) was added to a stirred solution of per-*O*-acetylated glycoside **42** (0.0144 g, 7.57 μmol) in dry methanol (10 mL) at RT under argon. The reaction was stirred for 18 h, neutralized with the addition of Dowex cation- exchange resin (H^+ ionic form), filtered, and the solution was concentrated under reduced pressure to dryness. The residue was subjected to reverse-phase (C-18) HPLC in water:methanol to afford azide **43** as a white solid (0.0022 g, 26%). $[\alpha]^{22}_D = -60.9°$ (*c* 0.22, CH_3OH); ^1H NMR (D_2O, 500 MHz): δ 5.90 (ddd, ^3J = 6.8 Hz, 10.5 Hz, 17.3 Hz, $CH_2=C\underline{H}$), 5.37 (dd, 1 H, ^2J < 1 Hz, J_{trans} = 17.3 Hz, CHaHb=CH), 5.32 (dd, 1 H, J_{cis} = 10.5 Hz, $CHa\underline{Hb}=CH$), 5.26 (d, 1 H, $J_{1,2}$ = 3.3 Hz, H-1’’’’’), 5.10 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1’’’), 4.86 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5’’’), ca. 4.72 (overlap with HOD, 1 H, H-1’’), 4.49 (d, 2 H, $J_{1,2}$ = 8.0 Hz, H-1, H-1’’’’), 4.43 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1’), 4.34 (dd, 1 H, ^3J = 4.7 Hz, 6.0 Hz, $C=CHC\underline{H}OH$), 4.23 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.5 Hz, H-5’’’’’), 4.13 (dd, 1 H, $J_{3,4}$ = 3.6 Hz, $J_{4,5}$ < 1 Hz, H-4’), 4.02-3.58 (m, 30 H, H-3, H-4, H-5, H-6a, H-6b, H-2’, H-3’, H-5’, H-6’a, H-6’b, H-2’’, H-3’’, H-4’’, H-6’’a, H-6’’b, H-2’’’, H-3’’’, H-4’’’, H-2’’’’, H-3’’’’, H-4’’’’, H-5’’’’, H-6’’’’a, H-6’’’’b, H-2’’’’’, H-3’’’’’, H-4’’’’’, $OC\underline{H_2}C\underline{H}N_3$), 3.44 (ddd, 1 H, $J_{4,5}$ = 9.6 Hz, $J_{5,6}$ = 2.1 Hz, 4.9 Hz, H-5’’), 3.34 (m, 1 H, higher order, H-2), 2.02 (s, 3 H, Ac), 1.26 (d, 3 H, H-6’’’’’), 1.23 (d, 3 H, H-6’’’); ^{13}C NMR (D_2O, 125 MHz): δ 135.9, 119.4, 103.7, 103.3, 102.8, 101.0, 100.2, 99.4, 82.8, 79.1, 77.2, 76.2, 75.7, 75.6, 75.2, 74.4, 73.9, 73.6, 73.0, 72.8, 72.6, 70.8, 70.6, 70.0, 69.6, 69.5, 69.1, 68.6, 67.8, 67.6, 65.8, 62.3, 61.8, 22.9, 16.4, 16.4; MS (ES) calcd. for $C_{43}H_{72}N_4O_{30}Na$ (M + Na) 1147.4, found *m/z* 1147.4.

(2S,3R)-2-butyramido-3-hydroxy-4-pentenyl α-L-fucopyranosyl-(1→ 2)-β-D-galacto-

pyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-gluco-pyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside (**44**)

Hydrogen sulfide gas was bubbled through a stirred solution of azide **43** (0.0048 g, 4.3 μmol) in a mixture of pyridine (10 mL), water (1 mL), and triethylamine (0.5 mL) at 0 °C for 24 h. The reaction progress (Rf_{43} = 0.6, Rf_{amine} = 0.5) was monitored by TLC using an eluent mixture of acetic acid: water: methanol: chloroform (1:2:5:4). The solvent was evaporated under reduced pressure. The residue was taken up in methanol, filtered through a cotton plug and Millex filter unit (0.45 μm), and the methanolic solution concentrated under reduced pressure. The residue was dissolved in dry methanol (1 mL), the solution was cooled to 0 °C, and butyric anhydride (0.02 mL, 0.1 mmol) was added. The ice bath was removed and the solution was stirred for 18 h at RT. Reverse-phase flash chromatography on two Sep-Pak (C-18) cartridges fitted with a Millex filter unit (0.45 μm) using water:methanol gave ceramide analog **44** as a white solid (0.0044 g, 88%). $[\alpha]^{22}_{D}$ = -35.6° (*c* 0.09, CH_3OH); ^{1}H NMR (D_2O, 600 MHz): δ 5.87 (ddd, 1 H, ^3J = 7.0 Hz, 10.6 Hz, 17.4 Hz, CHaHb=C$\underline{H}$), 5.34 (dd, 1 H, ^2J = 0.7 Hz, ^3J = 17.4 Hz, C$\underline{Ha}$Hb=CH), 5.30-5.27 (m, 2 H, CHa$\underline{Hb}$=CH, H-1'''''), 5.13 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1'''), 4.88 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5'''), ca. 4.74 (overlap with HOD, 1 H, H-1''), 4.52 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1''''), 4.49 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1), 4.45 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1'), 4.29-4.22 (m, 2 H, H-5''''', CH_2=CHC$\underline{H}$OH), 4.15 (dd, 1 H, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ < 1 Hz, H-4'), 4.12 (ddd, 1 H, ^3J = 3.3 Hz, 6.2 Hz, 6.2 Hz, H_2C=CHCH(OH)C$\underline{H}$), 4.07 (dd, 1 H, ^2J = 10.6 Hz, ^3J = 6.2 Hz, OC$\underline{Ha}$HbCHNH), 4.03-3.58 (m, 28 H, OCHa$\underline{Hb}$CHNH, H-3, H-4, H-5, H-6a, H-6b, H-2', H-3', H-5', H-6'a, H-6'b, H-2'', H-3'', H-4'', H-6''a, H-6''b, H-2''', H-3''', H-4''', H-2'''', H-3'''', H-4'''', H-5'''', H-6''''a, H-6''''b, H-2''''', H-3''''', H-4'''''), 3.47 (ddd, 1 H, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 2.8 Hz, 4.4 Hz, H-5''), 3.35 (dd, 1 H, $J_{2,3}$ = 8.4 Hz, H-2), 2.24 (t, 2 H, ^3J = 7.3 Hz, C(O)C$\underline{H_2}$$CH_2CH_3$), 2.04 (s, 3 H, Ac), 1.60 (tq, 2 H, ^3J = 7.3 Hz, 7.3 Hz, C(O)CH_2C$\underline{H_2}$$CH_3$), 1.28 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6'''''), 1.25 (d, 3 H, H-6'''), 0.91 (t, 3

H, $C(O)CH_2CH_2C\underline{H}_3$); selected ^{13}C HMQC NMR data (D_2O, 150 MHz): δ 119.0, 104.0, 103.5, 103.2, 101.1, 100.2, 99.5, 38.6, 23.1, 19.8, 16.2, 13.8; MS (ES) calcd. for $C_{47}H_{80}N_2O_{31}Na$ (M + Na) 1191.5, found *m/z* 1191.3.

(2S,3R)-8-amino-2-butyramido-3-hydroxy-6-thia-octyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside, acetate salt **(45)**

2,2’-azobis(2-methylpropionamidine) dihydrochloride (0.0095 g, 35 μmol) was added to a stirred solution of alkene **44** (0.0076 g, 6.5 μmol) and cysteamine hydrochloride (0.0300 g, 0.26 mmol) in degassed water (0.6 mL) at 60°C under argon. The reaction was stirred for 42 h and then quenched by addition of cyclohexene (0.2 mL, 2 mmol). The solution was concentrated to dryness under reduced pressure and subjected to size exclusion chromatography (Bio-Gel P4). The fractions containing the product were collected, concentrated, and subjected to reverse phase (C-8) HPLC in 0.3% acetic acid in water:methanol. The acetic acid salt of amine **45** was collected along with impurities (cysteamine derivatives) that could not be separated (0.0031 g, ca. 62% pure by NMR, ca. 23% yield.) ^1H NMR (D_2O, 600 MHz): δ 5.26 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1’’’’’), 5.10 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1’’’), 4.86 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.5 Hz, H-5’’’), ca. 4.71 (overlap with HOD, 1 H, H-1’’), 4.49 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1’’’’), 4.46 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1), 4.42 (d, 1 H, $J_{1,2}$ = 9.2 Hz, H-1’), 4.23 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5’’’’’), 4.13 (dd, 1 H, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ < 1 Hz, H-4’), 4.08 (dd, 1 H, ^2J = 10.5 Hz, ^3J = 6.0 Hz, H-*A*a), 4.04-3.55 (m, 30 H, H-3, H-4, H-5, H-6a, H-6b, H-2’, H-3’, H-5’, H-6’a, H-6’b, H-2’’, H-3’’, H-4’’, H-6’’a, H-6’’b, H-2’’’, H-3’’’, H-4’’’, H-2’’’’, H-3’’’’, H-4’’’’, H-5’’’’, H-6’’’’a, H-6’’’’b, H-2’’’’’, H-3’’’’’, H-4’’’’’, H-*A*b, H-*B*, H-*C*), 3.46-3.42 (m, 1 H, H-5’’), 3.31 (dd, 1 H, $J_{2,3}$ = 8.5 Hz, H-2), 3.21 (t, 2 H, ^3J = 6.7 Hz, H-*G*a, H-*G*b), 2.84 (ddd, 2 H, ^2J = 2.9 Hz, H-*F*a, H-*F*b), 2.74 (ddd, 1 H, ^2J = 13.3 Hz, ^3J = 4.6 Hz, 8.7

Hz, H-*E*a), 2.63 (ddd, 1 H, ^{3}J = 8.1 Hz, 8.1 Hz, H-*E*b), 2.24 (t, 2 H, ^{3}J = 7.3 Hz, H-*H*), 2.01 (s, 3 H, Ac), 1.86-1.80 (m, 1 H, H-*D*a), 1.74-1.68 (m, 1 H, H-*D*b), 1.60 (tq, 2 H, ^{3}J = 7.3 Hz, H-*I*), 1.25 (d, 3 H, H-6'''''), 1.22 (d, 3 H, H-6'''), 0.90 (t, 3 H, H-*J*); selected ^{13}C HMQC NMR data (D_2O, 150 MHz): δ 103.8, 103.3, 103.1, 101.0, 100.2, 99.4, 76.2, 73.8, 67.6, 67.7, 69.1, 38.6, 23.0, 20.0, 16.3, 13.7; MS (ES) calcd. for $C_{49}H_{87}N_3O_{31}SNa$ (M + Na) 1268.5, found *m/z* 1268.5.

(2S,3R)-2-butyramido-3-hydroxy-8-(β-maleimidopropionamido)-6-thia-octyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside (46)

A solution of 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (0.180 M) was freshly prepared in DMF (dried over 4 Å MS and pumped on high vacuum for 2 h). This solution (40 μL) was added to a stirred solution of amine **45** (0.0031 g, 2.4 μmol) in PBS (0.42 mL, 0.1 M sodium phosphate, 0.15 M NaCl, 5 mm EDTA, pH 7.2) at RT. After 4 h of stirring, the reaction was diluted to 10 mL with water and passed through a pre-washed Sep-Pak (C-18) cartridge and Millex filter unit (0.45 μm). The cartridge was washed with water (10 mL) and the product was eluted with methanol (20 mL). The solvent was evaporated under reduced pressure and the residue lyophilized from water to afford maleimide **46** as a white powder (0.0018 g, 54%). ^{1}H NMR (D_2O, 600 MHz): δ 6.86 (s, 2 H, maleimide), 5.27 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1'''''), 5.11 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1'''), 4.87 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5'''), ca. 4.73 (overlap with HOD, 1 H, H-1''), 4.52-4.42 (m, 3 H, H-1'''', H-1, H-1'), 4.25 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5'''''), 4.16-3.56 (m, 34 H, H-3, H-4, H-5, H-6a, H-6b, H-2', H-3', H-4', H-5', H-6'a, H-6'b, H-2'', H-3'', H-4'', H-6''a, H-6''b, H-2''', H-3''', H-4''', H-2'''', H-3'''', H-4'''', H-5'''', H-6''''a, H-6''''b, H-2''''', H-3''''', H-4''''', H-*A*a, H-*A*b, H-*B*, H-*C*, H-*L*a, H-*L*b), 3.47-3.43 (m, 1 H, H-5''), 3.36-3.31 (m, 3 H, H-2, H-*G*a, H-*G*b), 2.74-2.67 (m, 1 H, H-

*E*a), 2.64-2.56 (m, 3 H, H-*F*a, H-*F*b, H-*E*b), 2.54-2.51 (m, 2 H, H-*K*), 2.26 (m, 2 H, H-*H*), 2.03 (s, 3 H, Ac), 1.86-1.78 (m, 1 H, H-*D*a), 1.72-1.66 (m,1 H, H-*D*b), 1.61 (tq, 2 H, ^3J = 7.5 Hz, 7.5 Hz, H-*I*), 1.26 (d, 3 H, H-6''''), 1.23 (d, 3 H, H-6'''), 0.91 (t, 3 H, H-*J*).

BSA Derivatives (**47**, **48**, **49**)

BSA (0.0190 g, 0.29 μmol) was dissolved in buffer A (1.9 mL, 50 mM sodium phosphate, 0.15 M NaCl, pH 8.0, degassed). To the BSA solution was added a freshly prepared solution of 2-iminothiolane hydrochloride in buffer A (1036 μL, 27.6 mM). The reaction tube was tumbled at RT for 2 h. The reaction mixture was transferred to a Microsep Microconcentrator (10K MWCO) and subjected to repetitive (5 times) cycles of dilution with buffer B (0.1 M sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 7.2, degassed) and diafiltration. Following the last diafiltration step, the solution of thiolated BSA **47** was diluted to about 3 mL. To 2 mL of the solution of thiolated BSA **47** was added pentasaccharide Le^y derivative **39** (0.0036 g in 0.2 mL buffer B, 15 equivalents); to the remaining 1 mL of the solution of thiolated BSA **47** was added hexasaccharide Le^y derivative **46** (0.0018 g in 0.2 mL buffer B, 14 equivalents). The solutions were tumbled for 10 hr at RT and then stored at 4 °C for 7 h. The solutions of BSA conjugates **48** and **49** were then dialyzed (12-14K MWCO) against 5 changes of water (2L) at 4 °C and then lyophilized to give white solids. The average molecular weight for unmodified BSA, thiolated BSA **47**, glyconconjugate **48**, and glycoconjugate **49** as determined by MALDI mass spectral analysis were 66425, 74373, 79538 and 79445, respectively. Glycoconjugate **49** showed the greatest dispersion in molecular weight compared to BSA derivatives **47** or **48**.

11-azido-undecyl 2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-β-D-galactopyranoside (50)

A mixture of trichloroacetimidate **34** (0.0078 g, 5.1 μmol), 11-azido-undecanol (0.0093 g, 43.6 μmol), crushed 4 Å MS (0.43 g), dry dichloromethane (0.4 mL), and trimethylacetonitrile (0.8 mL) was stirred under argon at RT for 3 h and then cooled to 0 °C. Boron trifluoride diethyl etherate (12 μL, 95 μmol) was added dropwise to the reaction at 0 °C. The reaction was stirred for 3 days at RT and then quenched by addition of excess triethylamine at 0 °C. After 4 h of stirring, the mixture was filtered through Celite and the solvent evaporated under reduced pressure. The residue was subjected to column chromatography in toluene:acetone (2:1) to furnish glycoside **50** as a white powder (0.0038 g, 47%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ 5.43 (d, 1 H, $J_{NH,2}$ = 7.5 Hz, NH), 5.37 (d, 1 H, $J_{3,4}$ = 3.6 Hz, H-4), 5.34-5.30 (m, 4 H, H-1'''', H-4'', H-4''', H-4''''), 5.28 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1''), 5.16 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.3 Hz, H-3''), 5.12-5.09 (m, 2 H, H-3'''', H-1'), 5.06 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.1 Hz, H-2), 5.03 (dd, 1 H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 3.6 Hz, H-3'''), 5.00-4.92 (m, 4 H, H-2'''', H-6'a, H-2'', H-5''), 4.58 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1'''), 4.48 (dd, 1 H, ^2J = 11.4 Hz, $J_{5,6}$ = 5.8 Hz, H-6'''a), 4.45-4.38 (m, 2 H, H-5'''', H-3'), 4.37 (d, 1 H, H-1), 4.26 (dd, 1 H, $J_{5,6}$ = 8.4 Hz, H-6'''b), 4.12 (dd, 1 H, ^2J = 11.4 Hz, $J_{5,6}$ = 6.8 Hz, H-6a), 4.08-4.05 (m, 2 H, H-6b, H-6'b), 3.89-3.79 (m, 5 H, $N_3(CH_2)_{10}C\underline{Ha}HbO$, H-4', H-5''', H-3, H-5), 3.77 (dd, 1 H, H-2'''), 3.47-3.41 (m, 2 H, $N_3(CH_2)_{10}CHa\underline{Hb}O$, H-5'), 3.24 (t, 2 H, ^3J = 7.1 Hz, CH_2N_3), 2.92-2.86 (m, 1 H, H-2'), 2.16 (s, 3 H, Ac), 2.14 (s, 3 H, Ac), 2.13 (s, 3 H, Ac), 2.12 (s, 3 H, Ac), 2.11 (s, 3 H, Ac), 2.10 (s, 3 H, Ac), 2.08 (s, 3 H, Ac), 2.07 (s, 3 H, Ac), 2.05 (s, 3 H, Ac), 1.99 (s, 3 H, Ac), 1.97 (s, 3 H, Ac), 1.97 (s, 3 H, Ac), 1.96 (s, 3 H, Ac), 1.90 (s, 3 H, Ac), 1.60-1.48 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2N_3$), 1.38-1.23 (m, 14 H, $O(CH_2)_2(C\underline{H_2})_7(CH_2)_2N_3$), 1.20 (d, 3 H, $J_{5,6}$ = 6.5 Hz, H-6''''), 1.16 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’).

11-azido-undecyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside **(51)**

Sodium metal (ca. 2 mg, 87 μmol) was added to a stirred solution of glycoside **50** (0.0038 g, 2.4 μmol) in dry methanol (5 mL) at RT under argon. The reaction was stirred for 18 h and then neutralized with Dowex cation exchange resin (H^+ ionic form). The mixture was filtered through a cotton plug and the solvent evaporated under reduced pressure. The residue was subjected to reverse-phase (C-18) HPLC in water:methanol to afford de-*O*-protected glycoside **51** as a white solid (0.0008 g, 32%). $[\alpha]^{22}_D$ = -82.5° (*c* 0.08, CH_3OH); ^{1}H NMR (D_2O, 500 MHz): δ 5.25 (d, 1 H, $J_{1,2}$ = 3.0 Hz, H-1’’’’), 5.10 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1’’), 4.86 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5’’), ca. 4.74 (overlap with HOD, 1 H, H-1’), 4.50 (d, 1 H, $J_{1,2}$ = 7.3 Hz, H-1’’’), 4.35 (d, 1 H, $J_{1,2}$ = 8.2 Hz, H-1), 4.23 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.9 Hz, H-5’’’’), 4.12 (dd, 1 H, $J_{3,4}$ = 2.4 Hz, $J_{4,5}$ < 1 Hz, H-4), 4.02-3.58 (m, 23 H, H-3, H-5, H-6a, H-6b, H-2’, H-3’, H-4’, H-6’a, H-6’b, H-2’’, H-3’’, H-4’’, H-2’’’, H-3’’’, H-4’’’, H-5’’’, H-6’’’a, H-6’’’b, H-2’’’’, H-3’’’’, H-4’’’’, $N_3(CH_2)_{10}C\underline{Ha}HbO$, $N_3(CH_2)_{10}CHa\underline{Hb}O$), 3.53 (dd, 1 H, $J_{2,3}$ = 8.8 Hz, H-2), 3.48-3.42 (m, 1 H, H-5’), 3.29 (t, 2 H, ^3J = 6.9 Hz, CH_2N_3), 2.02 (s, 3 H, Ac), 1.65-1.56 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2N_3$), 1.40-1.24 (m, 17 H, $O(CH_2)_2(C\underline{H_2})_7(CH_2)_2N_3$, H-6’’’’), 1.23 (d, 3 H, H-6’’); ^{13}C NMR (D_2O, 150 MHz): δ 103.7, 103.1, 101.0, 100.2, 99.4, 83.3, 77.3, 76.2, 75.8, 75.4, 74.4, 74.0, 72.7, 72.4, 70.7, 69.9, 69.6, 69.1, 68.6, 68.4, 67.8, 67.5, 62.5, 61.4, 60.7, 52.2, 29.6, 29.3 28.7, 23.0, 16.2; MS (ES) calcd. for $C_{43}H_{76}N_4O_{24}Na$ (M + Na) 1055.5, found *m/z* 1055.

11-amino-undecyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fuco-pyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galacto-pyranoside (**52**)

Hydrogen sulfide was bubbled through a stirred solution of azide **51** (0.0037 g, 3.6 μmol) in a mixture of pyridine (10 mL), water (1 mL), and triethylamine (0.5 mL) at 0 °C. After 3 days, the solution was concentrated to dryness under reduced pressure. The residue was taken up in water and passed through a pre-washed Sep-Pak (C-18) cartridge. The cartridge was washed with water (20 mL) and the product eluted with 0.5 % acetic acid in methanol (40 mL). The solvent was evaporated under reduced pressure and co-evaporated three times with toluene to give amine **52** as a white solid (0.0012 g, 33%). $[\alpha]^{22}_{D}$ = -85.0° (*c* 0.12, CH_3OH); ^1H NMR (D_2O, 600 MHz): δ 5.28 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1''''), 5.11 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1''), 4.87 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.7 Hz, H-5''), ca. 4.73 (overlap with HOD, 1 H, H-1'), 4.51 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1'''), 4.38 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1), 4.25 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5''''), 4.13 (dd, 1 H, $J_{3,4}$ = 3.2 Hz, $J_{4,5}$ < 1 Hz, H-4), 4.00 (dd, 1 H, ^2J = 10.6 Hz, $J_{5,6}$ = 1.6 Hz, H-6'a), 3.96-3.63 (m, 21 H, $(CH_2)_{10}\underline{CH_2}O$, H-3, H-5, H-6a, H-6b, H-2', H-3', H-4', H-6'b, H-2'', H-3'', H-4'', H-2''', H-3''', H-4''', H-6'''a, H-6'''b, H-2'''', H-3'''', H-4''''), 3.60 (ddd, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 4.3 Hz, 7.7 Hz, H-5'''), 3.54 (dd, 1 H, $J_{2,3}$ = 8.3 Hz, H-2), 3.45 (ddd, 1 H, $J_{4,5}$ = 9.0 Hz, $J_{5,6}$ =1.6 Hz, 4.9 Hz, H-5'), 2.99 (t, 2 H, ^3J = 7.5 Hz, $\underline{CH_2}NH_2$), 2.03 (s, 3 H, Ac), 1.68-1.58 (m, 4 H, $\underline{CH_2}CH_2NH_2$, $\underline{CH_2}CH_2O$), 1.39-1.29 (m, 14 H, $H_2N(CH_2)_2(\underline{CH_2})_7(CH_2)_2O$), 1.27 (d, 3 H, H-6''''), 1.24 (d, 3 H, H-6''); ^{13}C NMR (D_2O, 150 MHz): δ 103.7, 103.3, 101.0, 100.2, 99.4, 83.4, 77.2, 76.3, 75.7, 75.3, 74.4, 73.9, 72.7, 71.6, 71.4, 70.6, 70.3, 69.9, 69.5, 69.1, 68.6, 67.7, 67.5, 62.5, 62.4, 61.5, 61.3, 60.5, 40.3, 29.4, 29.3, 27.2, 26.0, 23.0, 16.2; HRMS (ES) calcd. for $C_{43}H_{79}N_2O_{24}$ (M + H) 1007.5023, found *m/z* 1007.5024.

1-[11-amino-undecyl α-L-fucopyranosyl-(→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fuco-

pyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranoside]-2-ethoxycyclobutene-3,4-dione **(53)**

Ethanolic diethyl squarate (6.7 μL, 0.17 M, 0.95 equivalent) was added to a stirred solution of amine **52** (0.0012 g, 1.2 μmol) in 1.0 mL PBS (20 mM sodium phosphate, 0.15 M NaCl, 1mM EDTA, pH 7.2) at RT. After 18 h, the reaction solution was diluted to 5 mL with PBS and passed through a pre-washed Sep-Pak (C-18) cartridge and Millex filter unit (0.45 μm). The cartridge was washed with water (10 mL) and the product eluted with methanol (30 mL). The solvent was evaporated under reduced pressure to yield squarate derivative **53** as a white solid. Selected ^{1}H NMR data (CD_3OD, 500 MHz): δ 5.14 (d, 1 H, $J_{1,2}$ = 3.5 Hz, H-1''''), 5.03 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1''), 1.96 (s, 3 H, NAc), 1.64-1.54 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2NH$), 1.46-1.42 (m, 3 H, $OCH_2C\underline{H_3}$), 1.39-1.25 (m, 14 H, $O(CH_2)_2(C\underline{H_2})_7(CH_2)_2NH$), 1.22 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6''''), 1.21 (d, 3 H, $J_{5,6}$ = 6.5 Hz, H-6'').

Pentasaccharide Le^y HHb Glycoconjugate **(54)**

A solution of squarate derivative **53** (ca. 1.2 μmol) in 3 mL borate buffer (0.35 M $KHCO_3$, 0.08 M $Na_2B_4O_7$, pH 9.02) was added to horse hemoglobin (0.0074 g, 0.12 μmol). The reaction was tumbled for 20 h at RT. The solution was dialyzed against 5 changes of water (2L) at 4 °C and then lyophilized to afford conjugate **54** as a red solid. MALDI mass spectral analysis of glycoconjugate **54** confirmed that derivatization of native HHb had been achieved.

11-azido-undecyl 2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 2)-3,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-[2,3,4-tri-O-acetyl-α-L-fucopyranosyl-(1→ 3)]-2-acetamido-6-O-acetyl-2-deoxy-β-D-glucopyranosyl-(1→ 3)-2,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→ 4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside **(55)**

A mixture of trichloroacetimidate **41** (0.0149 g, 8.2 μmol), 11-azido-undecanol (0.0049 g, 23 μmol), crushed 4 Å MS (0.7 g), and dry dichloromethane (1 mL) was stirred at RT under argon for 3 h. The mixture was cooled to 0 °C and boron trifluoride diethyl etherate (15 μL, 0.12 mmol) was added dropwise. The reaction was stirred at 0 °C under argon for 48 h, diluted with dichloromethane, and filtered through Celite. The solution was washed sequentially with aqueous sodium bicarbonate, water, and brine and then dried over anhydrous sodium sulphate. The solvent was evaporated under reduced pressure and the residue was subjected to column chromatography in toluene:acetone (2:1) to afford glycoside **55** as a white powder (0.0025 g, 16%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.38 (d, 1 H, $J_{NH,2}$ = 6.7 Hz, $N\underline{H}Ac$), 5.34-5.28 (m, 5 H, H-1’’’’’, H-4’’’, H-4’’’’, H-4’, H-4’’’’’), 5.23 (d, 1 H, $J_{1,2}$ = 3.8 Hz, H-1’’’), 5.18-5.13 (m, 2 H, H-3, H-3’’’), 5.08 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.1 Hz, H-3’’’’’), 5.06 (d, 1 H, $J_{1,2}$ = 8.6 Hz, H-1’’), 5.05-4.92 (m, 6 H, H-6’’a, H-3’’’’, H-2’’’’’, H-2’, H-2’’’, H-5’’’), 4.84 (dd, 1 H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 4.58 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1’’’’), 4.52- 4.46 (m, 2 H, H-6a, H-6’’’’a), 4.43-4.40 (m, 3 H, H-1, H-1’, H-5’’’’’), 4.35 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 9.5 Hz, H-3’’), 4.24 (dd, 1 H, ^2J = 11.6 Hz, $J_{5,6}$ = 8.6 Hz, H-6’’’’b), 4.10-3.98 (m, 4 H, H-6b, H-6’a, H-6’’b, H-6’b), 3.89-3.81 (m, 2 H, H-4’’, H-5’’’’), 3.81-3.74 (m, 5 H, H-3’, $OC\underline{Ha}Hb(CH_2)_{10}N_3$, H-5’, H-4, H-2’’’’), 3.55 (ddd, 1 H, $J_{4,5}$ = 9.9 Hz, $J_{5,6}$ = 1.8 Hz, 4.7 Hz, H-5), 3.43-3.39 (m, 2 H, $OCHa\underline{Hb}(CH_2)_{10}N_3$, H-5’’), 3.22 (t, 2 H, ^3J = 7.0 Hz, CH_2N_3), 2.85 (m, 1 H, broad, H-2’’), 2.15 (s, 3 H, Ac), 2.13 (s, 6 H, 2 Ac), 2.12 (s, 3 H, Ac), 2.10 (s, 6 H, 2 Ac), 2.09 (s, 6 H, 2 Ac), 2.07 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.01 (s, 3 H, Ac), 2.00 (s, 3 H, Ac), 1.99 (s, 3 H, Ac), 1.97 (s, 6 H, 2 Ac), 1.96 (s, 3 H, Ac), 1.90 (s, 3 H, Ac), 1.66-1.54 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2N_3$), 1.36-1.22 (m, 14 H, $O(CH_2)_2(C\underline{H_2})_7(CH_2)_2N_3$), 1.19 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’’’), 1.15 (d, 3 H, $J_{5,6}$ = 6.6 Hz, H-6’’’); MS (ES) calcd. for $C_{81}H_{119}N_4O_{45}$ (M + H) 1867.7, found *m/z* 1867.6.

11-azido-undecyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galacto-

pyranosyl-(1→ 4)-β-D-glucopyranoside (**56**)

Sodium metal (ca. 2 mg, 87 μmol) was added to a solution of per-*O*-acetylated glycoside **55** (0.0040 g, 2.1 μmol) in dry methanol (5 mL) under argon and the solution was stirred at RT for 18 h. The solution was then neutralized with Dowex cation exchange resin (H^+ ionic form), and the mixture was filtered through a cotton plug. Evaporation of the solvent under reduced pressure afforded glycoside **56** in quantitative yield as estimated from TLC. ^1H NMR (CD_3OD, 400 MHz): δ 5.14 (d, 1 H, $J_{1,2}$ = 3.4 Hz, H-1’’’’’), 5.03 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1’’’), 4.83 (m, 1 H, H-5’’’), 4.66 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1’’), 4.50 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1’’’’), 4.34 (d, 1 H, $J_{1,2}$ = 7.3 Hz, H-1’), 4.25 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1), 4.15 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5’’’’’), 4.02 (dd, 1 H, $J_{3,4}$ = 2.8 Hz, $J_{4,5}$ < 1 Hz, H-4’), 3.95-3.18 (m, 33 H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2’, H-3’, H-5’, H-6’a, H-6’b, H-2’’, H-3’’, H-4’’, H-5’’, H-6’’a, H-6’’b, H-2’’’, H-3’’’, H-4’’’, H-2’’’’, H-3’’’’, H-4’’’’, H-5’’’’, H-6’’’’a, H-6’’’’b, H-2’’’’’, H-3’’’’’, H-4’’’’’, $\mathrm{OC\underline{Ha}Hb(CH_2)_{10}N_3}$, $\mathrm{OCHa\underline{Hb}(CH_2)_{10}N_3}$, $\mathrm{C\underline{H_2}N_3}$), 1.95 (s, 3 H, Ac), 1.62-1.52 (m, 4 H, $\mathrm{OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2N_3}$), 1.38-1.10 (m, 20 H, $\mathrm{O(CH_2)_2(C\underline{H_2})_7(CH_2)_2N_3}$, H-6’’’’’, H-6’’’); MS (ES) calcd. for $C_{49}H_{86}N_4O_{29}Na$ (M + Na) 1217.5, found *m/z* 1217.5.

11-amino-undecyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside (**57**)

Hydrogen sulfide gas was bubbled through a stirred solution of azide **56** (0.0026 g, 2.1 μmol) in a mixture of pyridine (10 mL), water (1 mL), and triethylamine (0.5 mL) at 0 °C for 48 h. The conversion appeared to be quantitative as estimated from TLC. The solvent was evaporated under reduced pressure and the residue was taken up in methanol and filtered through a Millex filter unit (0.45 μm). The solvent was evaporated under reduced pressure. The residue was dissolved in water and passed through a pre-washed Sep-Pak (C-18). The product was eluted with 33% methanol in water and the solvent was evaporated under reduced pressure to furnish amine **57**. Although some non-carbohydrate impurities co-eluted with amine **57**, no further attempt at purification was carried out. ^{1}H NMR (CD_3OD, 600 MHz): δ 5.15 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1’’’’’), 5.05 (d, 1 H, $J_{1,2}$ = 4.0 Hz, H-1’’’), 4.85-4.77 (m, 1 H, H-5’’’), 4.69 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1’’), 4.51 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1’’’’), 4.37 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1’), 4.27 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1), 4.17 (dq, 1 H, $J_{4,5}$ < 1 Hz, $J_{5,6}$ = 6.6 Hz, H-5’’’’’), 4.04 (dd, 1 H, $J_{3,4}$ = 2.8 Hz, $J_{4,5}$ < 1 Hz, H-4’), 3.96-3.16 (m, 31 H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2’, H-3’, H-5’, H-6’a, H-6’b, H-2’’, H-3’’, H-4’’, H-5’’, H-6’’a, H-6’’b, H-2’’’, H-3’’’, H-4’’’, H-2’’’’, H-3’’’’, H-4’’’’, H-5’’’’, H-6’’’’a, H-6’’’’b, H-2’’’’’, H-3’’’’’, H-4’’’’’, $OC\underline{Ha}Hb(CH_2)_{10}NH_2$, $OCHa\underline{Hb}(CH_2)_{10}NH_2$), 2.94-2.88 (m, 2 H, $C\underline{H_2}NH_2$), 1.97 (s, 3 H, Ac), 1.70-1.50 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2NH_2$), 1.45-1.35 (m, 14 H, $O(CH_2)_2(C\underline{H_2})_7(CH_2)_2NH_2$), 1.24-1.18 (m, 6 H, H-6’’’’’, H-6’’’); HRMS (ES) calcd. for $C_{49}H_{88}N_2O_{29}Na$ (M + Na) 1191.5370, found *m/z* 1191.5378.

1-[11-amino-undecyl α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-[α-L-fucopyranosyl-(1→ 3)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→ 3)-β-D-galactopyranosyl-(1→ 4)-β-D-glucopyranoside]-2-ethoxycyclobutene-3,4-dione (**58**)

Diethyl squarate (34.6 μL, 0.37 M in ethanol) was added to a stirred solution of amine **57** (0.0047 g, 4.0 μmol) in 4 mL PBS (0.1 M sodium phosphate, pH 7.0) at RT. The solution was stirred at RT for 4 days and then subjected to reverse phase chromatography on a Sep-Pak (C-18) cartridge. The product eluted in 50% methanol:water. The solvent was evaporated under reduced pressure to furnish squarate derivative **58** as a white solid. Selected ^{1}H NMR data (CD_3OD, 400 MHz): δ 5.14 (d, 1 H, $J_{1,2}$ = 3.2 Hz, H-1’’’’’), 5.03 (d, 1 H, $J_{1,2}$ = 4.2 Hz, H-1’’’), 1.95 (s, 3 H, NAc), 1.64-1.54 (m, 4 H, $OCH_2C\underline{H_2}(CH_2)_7C\underline{H_2}CH_2NH$), 1.46-1.38 (m, 3 H, $OCH_2C\underline{H_3}$), 1.34-1.24 (m, 14 H, $OCH_2CH_2(C\underline{H_2})_7CH_2CH_2NH$), 1.21 (d, 3 H, $J_{5,6}$ = 6.3 Hz, H-6’’’’’), 1.20 (d, 3 H, $J_{5,6}$ = 6.7 Hz, H-6’’’); MS (ES) calcd. for $C_{55}H_{92}N_2O_{32}Na$ (M + Na) 1315.6, found *m/z* 1316.

Hexasaccharide Le^y HHb Glycoconjugate (**59**)

A solution of squarate derivative **58** (2.1 μmol) in borate buffer (4 mL, 0.35 M $KHCO_3$, 0.08 M $Na_2B_4O_7$, pH 9.02) was added to horse hemoglobin (0.0080 g, 0.13 μmol). The solution was tumbled for 16 h at RT, dialyzed against 6 changes of water (2L) at 4 °C, and then lyophilized to give conjugate **59** as a red solid. MALDI mass spectral analysis of glycoconjugate **59** confirmed that derivatization of native HHb had been achieved.

6.2. FAC/MS Assays

6.2.a. Materials

EZ-Link Sulfo-NHS-LC-Biotin was from Pierce. PEEK fingertight union, ferrules, and Hamilton Gastight syringes (1 mL model #1001) were obtained from Supelco. Reducing ferrules were from Alltech. PEEK tubing (0.50 mm ID and 0.13 mm ID), union, tee, and frit were from Fisher Scientific. CPG-SA beads were from CPG Inc., USA. Methoxycarbonyloctyl glycosides (**22**, **24**, and **25**) were gifts from Dr. O. Hindsgaul. Monoclonal antibody B3 was a gift from Dr. I. Pastan at NIH.

6.2.b. Biotinylation of Monoclonal Antibody B3

A solution of B3 in storage buffer (0.98 mg, 1 mL) was concentrated by diafiltration in a Microsep microconcentrator (10K MWCO) by centrifugation (1 h at 5000 g). Buffer C (0.250 mL, 50 mM sodium bicarbonate, 150 mM sodium chloride, pH 8.54) was added and diafiltration was repeated. The solution of B3 was transferred to an Eppendorf tube, diluted to ca. 500 μL with buffer C, and treated with an aqueous solution of Le^y methoxycarbonyloctyl derivative **22** (0.138 mL, 0.473 mM). After tumbling at 4 °C for 20 minutes, the B3 solution was treated with freshly-prepared, aqueous EZ-Link Sulfo-NHS-LC-Biotin (3 μL, 21.9 mM). The mixture was tumbled at 4 °C for another 5 minutes. The reaction was then cooled to 0 °C and quenched by addition of TRIS buffer (40 μL, 5 mM TRIS, 15 mM sodium chloride, pH 8.0). Concentration of the B3 solution via diafiltration through a MICROSEP microconcentrator (10K MWCO) was followed by dilution with PBS (300 μL, 10 mM sodium phosphate, 150 mM sodium chloride, 0.1 % NaN_3, pH 7.4) and a second diafiltration. PBS buffer (500 μL) was added and the biotinylated B3 solution was stored at 4 °C for later use.

6.2.c. Preparation of the microscale B3 affinity column

A 1 mL gas-tight Hamilton syringe was charged with a suspension of CPG-SA beads (ca. 100 μL, 37-74 μm) in aqueous glycerol (1 mL, 1:1 v/v). PEEK tubing (8 cm, 0.50 mm ID) was inserted directly into the Hamilton syringe in place of the needle, and the free end of the PEEK tubing was fitted with a ferrule, frit and mixing tee. Using a syringe pump (PHD 2000, Harvard Apparatus), the bead suspension was infused (30-80 μL/min) into the PEEK tubing with constant manual rotation of the Hamilton syringe to re-suspend the beads. Once packed with beads (by visual estimation), the PEEK tubing was removed from the syringe and the free end fitted with a ferrule, frit and PEEK union to constitute the complete column. Using the syringe pump and a Hamilton syringe with a blunt-tip needle, the column was washed (80 μL/min) with PBS (1 mL). The column was infused (8 μL/min) first with biotinylated B3 (PBS solution, ca. 6500 pmol) and then 1ml of Buffer D (10 mM ammonium acetate, 1 mM NaCl, pH 7.4). The remaining streptavidin sites were blocked by the infusion (8 μL/min) of D-biotin (0.2 mg in 1 mL PBS). Following a final wash with Buffer D (1 mL), the B3 affinity column was stored at 4 °C for later use.

6.2.d. Preparation of the blank FAC column for control experiments

A control column was prepared according to the same procedure used for the B3 affinity column (described above) except that infusion of biotinylated B3 through the column was omitted.

6.2.e FAC/MS Apparatus and Methods

The apparatus and protocol were described elsewhere.[88, 90, 91] Experiments were performed at RT at a flow rate of 8 μL/min. All solutions were prepared in Buffer D (10 mM ammonium acetate, 1 mM sodium chloride, pH 7.4). The Agilent Technologies

1100MSD (single quadrupole mass spectrometer) was programmed for selective ion monitoring of the sodium adducts of the carbohydrate analytes. The chromatograms presented in the figures (Chapter 3) were created using IGOR Pro software (Wavemetrics, Inc.).

6.3. Immunological Assays

6.3.a. Materials

BSA and HHb were from Sigma. Monoclonal antibody B3 was a gift from Dr. I. Pastan at NIH. The Le^y-BSA neoglycoprotein (Lemieux spacer) used in the B3 inhibition ELISA assays was obtained from Professors R. U. Lemieux and Hindsgaul. BALB/c mice were from Charles River Laboratories (Washington, MA). Anti-mouse IgG (whole molecule)-alkaline phosphatase from goat in buffered aqueous glycerol solution (A 3562), anti-mouse IgM (μ-chain specific)-alkaline phosphatase from goat in buffered aqueous solution (A 7784), and 4-nitrophenylphosphate disodium salt hexahydrate were from Sigma. Anti-mouse IgG goat antibody conjugated to horseradish peroxidase was from Kirkegaard and Perry Laboratories.

6.3.b. Inhibition ELISA screening of the reactivity of synthetic Le^y compounds with B3 (performed by J. Sadowska)

Le^y-BSA conjugate (100 μL, 1 μg/mL in PBS) was added to each well of a 96-well Nunc-Immuno ELISA plate (Maxisorp F96) and allowed to stand at 4 °C for 18 h. The plate was washed four times with PBST. Blocking was carried out by the addition of BSA (100 μL, 2% in PBS), which was allowed to stand for 1 h at RT. The plate was washed four times with PBST before the triplicate addition to the wells of a 1:1 v/v mixture of a synthetic ligand (**1**, **2**, **3**, or **22**, serially diluted in PBS) and monoclonal antibody B3 (0.08 μg/mL in PBS). The plate was equilibrated at RT for 18 h and then washed four times with PBST. Anti-mouse-IgG goat antibody conjugated to horseradish peroxidase (100 μL, 1:2000 dilution in PBS) was added to each each well and incubated for 1 h at RT. The plate was washed four times with PBST and then 3,3',5,5'-tetramethylbenzidine

(TMB, 100 μL) was added. The reaction was quenched after 2 minutes at RT by addition of 1 M phosphoric acid (100 μL) and the absorbance was read at 450 nm. The absorbance measurements were used to generate inhibition data.

6.3.c. Immunization of Mice with BSA Glycoconjugates 48 and 49 (performed by J. Sadowska)

Two groups of five retired breeder BALB/c mice were used for immunization experiments. One group was immunized with glycoconjugate **48**, the other with glycoconjugate **49**. On day 0, the mice were injected subcutaneously with 200 μL of a 2:1:1 mixture of the following 3 reagents: glycoconjugate solution (50 μg/mL in PBS), Freund's complete adjuvant, and Freund's incomplete adjuvant. On days 30 and 56, the mice were injected with 200 μL of a 1:1 mixture of glycoconjugate solution (50 μg/mL in PBS) and Freund's incomplete adjuvant. On day 66, the mice were sacrificed, exsanguinated, and the sera collected.

6.3.d. ELISA of Mice Sera for Reactivity with Le^y-HHb Glycoconjugates 54 and 59 (performed by J. Sadowska)

Glycoconjugates **54** and **59** were used to coat a 96-well Nunc-Immuno ELISA plate (MaxiSorp F96). To each well, a solution of glycoconjugate (100 μL, 10 μg/mL in PBS) was added and allowed to stand for 18 h at 4 °C. The plate was washed five times with PBST (PBS containing Tween 20, 0.05% v/v). 100 μL of serially diluted mouse sera (20, 100, 500, 2500, 12500, 62500) were added to the wells of the coated plate and incubated for 2 h at room temperature. The plate was washed four times with PBST, and either anti-mouse-IgG or anti-mouse-IgM goat antibody conjugated to alkaline phosphatase (100 μL, 1:2000 dilution in PBS) were added to the wells and equilibrated for 1 hr before washing four times with PBST. *p*-Nitrophenyl phosphate (100 μL, 1 mg/mL) in carbonate buffer (0.05 M, 1 mM magnesium chloride, pH 9.8) was added and after 1 hr at RT, the absorbance was read at 405 nm.

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Appendices

A.1. FAC/MS Data

Table A.1.1. *Infusion (8 µL/min) of compound 2 and void marker 23 (2 µM) in buffer (10 mM ammonium acetate, 1 mM sodium chloride, pH 7.42) through a B3 affinity column. The effluent was monitored by electrospray MS in SIM (m/z corresponding to M + Na) and positive ion mode.*

Concentration of Compound **2** (µM)	$V-V_o$, Retention volume (µL)
1	50.44
1	52.78
1	49.75
2	49.39
2	46.97
2	47.08
5	43.08
5	45.41
5	44.25
10	38.17
10	37.56
10	36.40
20	31.29
20	31.06
20	39.73

Table A.1.2. *Table of values obtained from linear regression analysis of the data shown in Table A.1.1.*

Equation 2:

$$\underbrace{\frac{1}{[2]_o\,(V-V_o)}}_{Y} = \underbrace{\frac{K_d}{B_t}}_{B} \bullet \underbrace{\frac{1}{[2]_o}}_{X} + \underbrace{\frac{1}{B_t}}_{A}$$

Parameter	Value	Error
A	777.63191	123.07627
B	18941.53782	233.03858
R	0.99909	

Table A.1.3. *Infusion (8 μL/min) of compound 3 and void marker 23 (2 μM) in buffer (10 mM ammonium acetate, 1 mM sodium chloride, pH 7.42) through a B3 affinity column. Effluent was monitored by electrospray MS in SIM (m/z corresponding to M + Na) and positive ion mode.*

Concentration of Compound **3** (μM)	V-V$_o$, Retention volume (μL)
1	122.61
1	119.17
1	120.70
2	111.52
2	111.88
2	114.06
5	89.56
5	88.60
5	85.98
10	68.36
10	68.49
10	67.49
20	52.60
20	47.91
20	51.18
20	50.73

Table A.1.4. *Table of values obtained from linear regression analysis of the data shown in Table A.1.3.*

Equation 2:

$$\underbrace{\frac{1}{[3]_o(V-V_o)}}_{Y} = \underbrace{\frac{K_d}{B_t}}_{B} \cdot \underbrace{\frac{1}{[3]_o}}_{X} + \underbrace{\frac{1}{B_t}}_{A}$$

Parameter	Value	Error
A	6.72223×10^{-4}	2.74634×10^{-5}
B	0.00761	5.55553×10^{-5}
R	0.99963	

Table A.1.5. *Infusion (8 µL/min) of compound 22 and void marker 23 (2 µM) in buffer (10 mM ammonium acetate, 1 mM sodium chloride, pH 7.42) through a B3 affinity column. Effluent was monitored by electrospray MS in SIM* (m/z *corresponding to M + Na) and positive ion mode.*

Concentration of Compound **22** (µM)	V-V$_0$, Retention volume (µL)
1	120.099
1	117.316
1	126.464
2	101.862
2	99.698
2	98.884
5	76.531
5	75.983
5	75.180
10	59.777
10	57.484
10	64.113
20	44.554
20	45.625
20	46.346

Table A.1.6. *Table of values obtained from the linear regression analysis of the data shown in Table A.1.5.*

Equation 2:

$$\underbrace{\frac{1}{[22]_o (V-V_o)}}_{Y} = \underbrace{\frac{K_d}{B_t}}_{B} \bullet \underbrace{\frac{1}{[22]_o}}_{X} + \underbrace{\frac{1}{B_t}}_{A}$$

Parameter	Value	Error
A	9.7282×10^{-4}	9.63896×10^{-5}
B	0.00745	1.88854×10^{-4}
R	0.99584	

Table A.1.7. *Table of break-through volumes obtained from duplicate FAC/MS analyses of a mixture of 2, 3, 22, and 23 each present at a concentration of 2 μM. These values were used to estimate* K_d^{mix} *for 3 and 22 from Equation 2 in two steps. First, the value of* B_t *was calculated from the breakthrough volumes for 2 and its independently-determined* K_d *of 24 ± 4 μM. Second, the value of* B_t *was used to estimate* K_d^{mix} *for 3 and 22.*

Compound	$V-V_o$, Breakthrough Volume (μL)	Variance in $V-V_o$ $(\mu L)^2$
2	55.48	0.15125
2	56.03	
3	102.07	0.0288
3	101.83	
22	108.79	0.31205
22	108.00	

Table A.1.8. *Table of break-through volumes obtained from FAC/MS analysis of a mixture of **1, 2, 3, 22,** and **23** each present at a concentration of 2 μM. These values were used to estimate K_d^{mix} for 1 from Equation 2 in two steps. First, the average value of B_t was calculated from the breakthrough volumes for **2, 3,** and **22,** and their independently-determined K_ds (24 ± 4 μM, 11.3 ± 0.5 μM, 7.7 ± 0.8 μmol, respectively). Subsequently, the average value of B_t was used to estimate K_d^{mix} for 1.*

Compound	V-V$_o$, Retention Volume (μL)
1	22.72
2	26.88
3	53.44
22	56.32

A.2. FAC/MS Error Analysis

The following equations were used to estimate K_d and B_t from FAC/MS data.

Definitions:

K_d is the dissociation constant describing the interaction of ligands A and B

$[A]_o$ is the infusion concentration of the analyte ligand A

$(V\text{-}V_o)$ is the retention volume of the analyte ligand A

V_o is the elution volume in the absence of specific adsorption

B_t is the binding capacity (total amount of immobilized ligand B)

From Kasai et al.[89]: $y = m\,x + b$

Where $y = 1/\left([A]_o (V\text{-}V_o)\right)$

$x = 1/[A]_o$

$m = K_d/B_t$

$b = 1/B_t$

A.2.a. Individually-determined K_d

Linear regression analysis of the FAC/MS data gives estimates for m (slope), b (y-intercept), and the standard deviations of m and b (S_b and S_m respectively). The variance of K_d and B_t (S_{Kd}^2 and S_{Bt}^2) were estimated by propagation of the errors in m and b:

$B_t = 1/b$

$$S^2_{B_t} = \left(\frac{\delta B_t}{\delta b}\right)^2 S_b^2 = \left(-b^{-2}\right)^2 S_b^2$$

$K_d = mB_t$

$$S^2_{K_d} = \left(\frac{\delta K_d}{\delta m}\right)^2_{B_t} S_m^2 + \left(\frac{\delta K_d}{\delta B_t}\right)^2_m S^2_{B_t} = B_t^2 S_m^2 + m^2 S^2_{B_t}$$

A.2.b. Determination of the Apparent K_d (in a mixture)

In our calculations, the variance in the analyte infusion concentration $[\text{analyte}]_0$ was assumed to be negligible. Also, the relative standard deviation in the retention volume V_R (where $V_R = V - V_0$) was estimated to be about 17%.

For a mixture of a void volume marker, and two ligands (A and B), both of known concentrations but only one (A) whose K_d is known:

1. The break-through volumes for both ligands (A and B) in the mixture were measured simultaneously in a single run.
2. The following equations were used to calculate the column capacity (B_t) by substituting the values for the known K_d and the measured retention volume V_R for the ligand (A) whose K_d is known:

$$B_t = (V_R)\,[A]_0 + (V_R)\,K_d$$

$$S^2_{B_t} = \left(\frac{d\,B_t}{d\,V_R}\right)^2_{[A]_0,\,K_d} S^2_{V_R} + \left(\frac{d\,B_t}{d[A]_0}\right)^2_{V_R,\,K_d} S^2_{[A]_0} + \left(\frac{d\,B_t}{d\,K_d}\right)^2_{V_R,[A]_0} S^2_{[A]_0}$$

$$S^2_{B_t} = \left[[A]_0 + K_d\right]^2 S^2_{V_R} + V_R^2\, S^2_{[A]_0} + V_R^2\, S^2_{[A]_0}$$

3. The following equations were used to calculate the apparent K_d for the other ligand (B) by substituting the value of B_t calculated in step (2) above and substituting the measured retention volume for ligand (B):

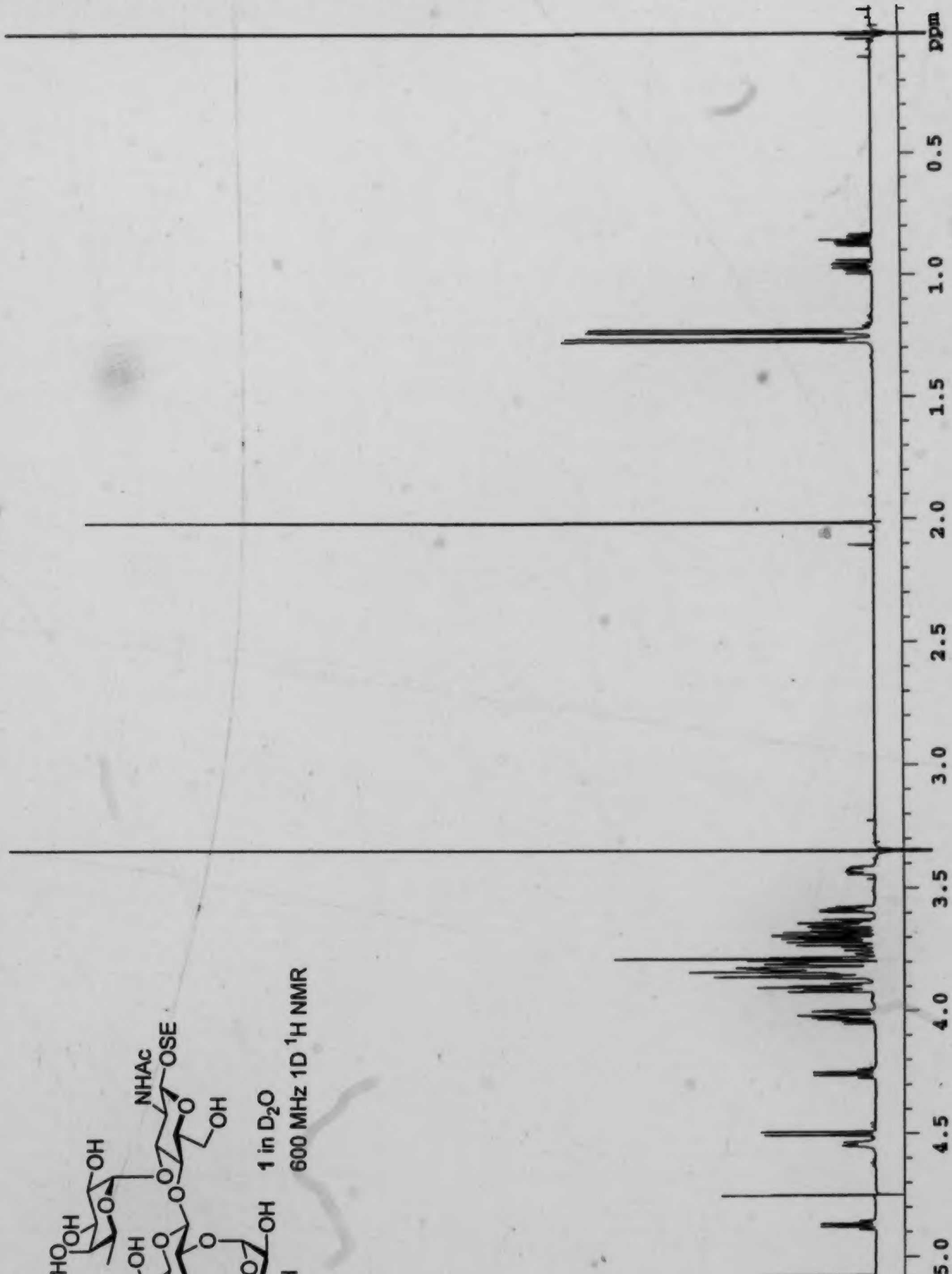
$$K_d = \frac{B_t}{V_R} - [B]_0$$

$$S^2_{K_d} = \left(\frac{d K_d}{d B_t}\right)^2_{V_R,[B]_0} S^2_{B_t} + \left(\frac{d K_d}{d V_R}\right)^2_{B_t,[B]_0} S^2_{V_R} + \left(\frac{d K_d}{d [B]_0}\right)^2_{V_R, B_t} S^2_{[B]_0}$$

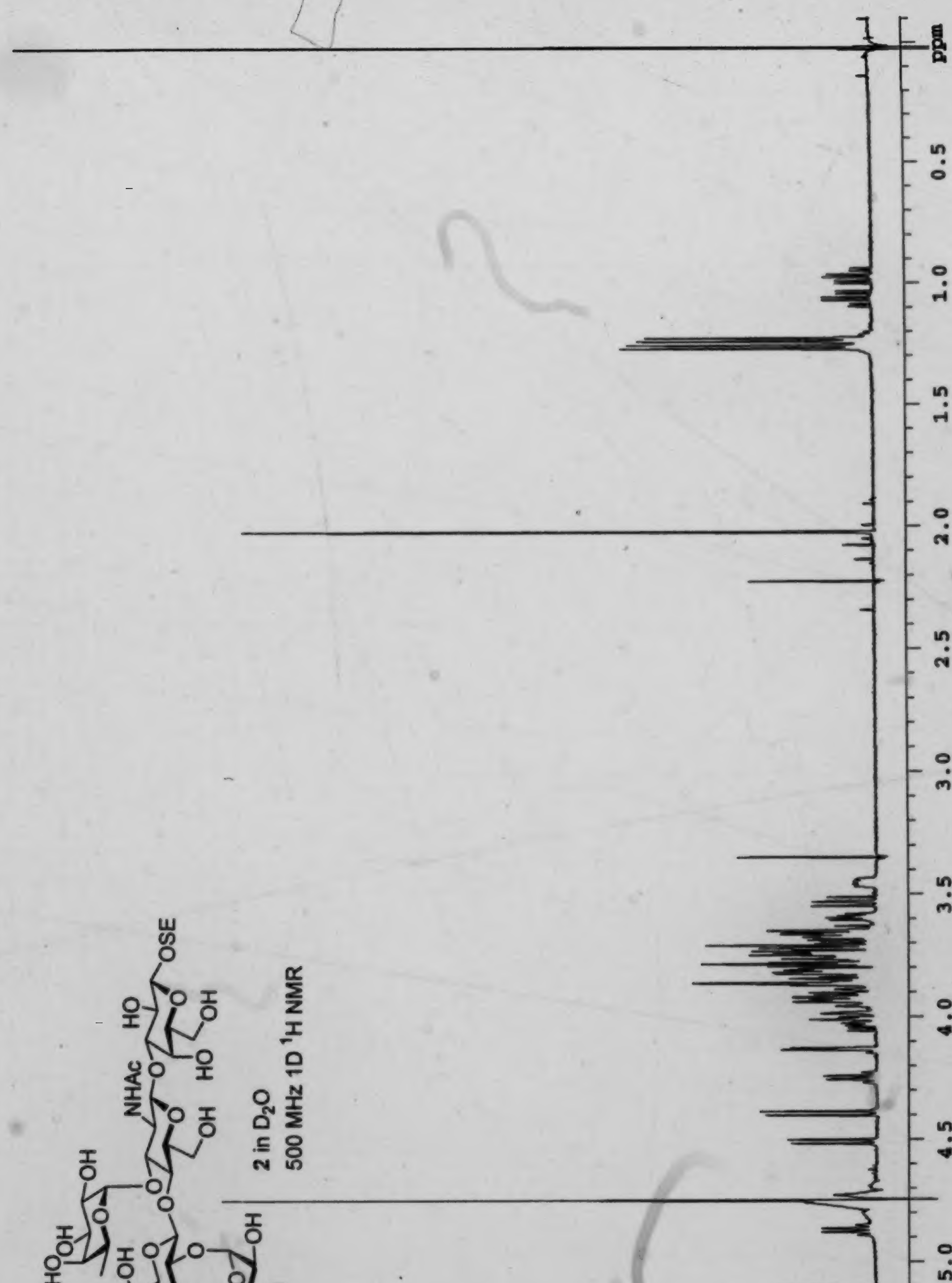
$$S^2_{K_d} = V_R^{-2} S^2_{B_t} + \left[-B_t (V_R)^{-2}\right]^2 S^2_{V_R} + (-1)^2 S^2_{[B]_0}$$

4. If the unknown ligand was run in a mixture with *several* other ligands whose K_ds were known, the apparent K_d (via B_t) was estimated by averaging the results obtained by performing the calculations (in step 2 and step 3) several times. (E.g. if one unknown ligand was run in a mixture with three ligands whose individually determined K_d values are known, the apparent K_d was calculated 3 times and then averaged. The relative standard deviation in the averaged value for the apparent K_d was taken to be equal to the greatest relative standard deviation calculated in step 3.)

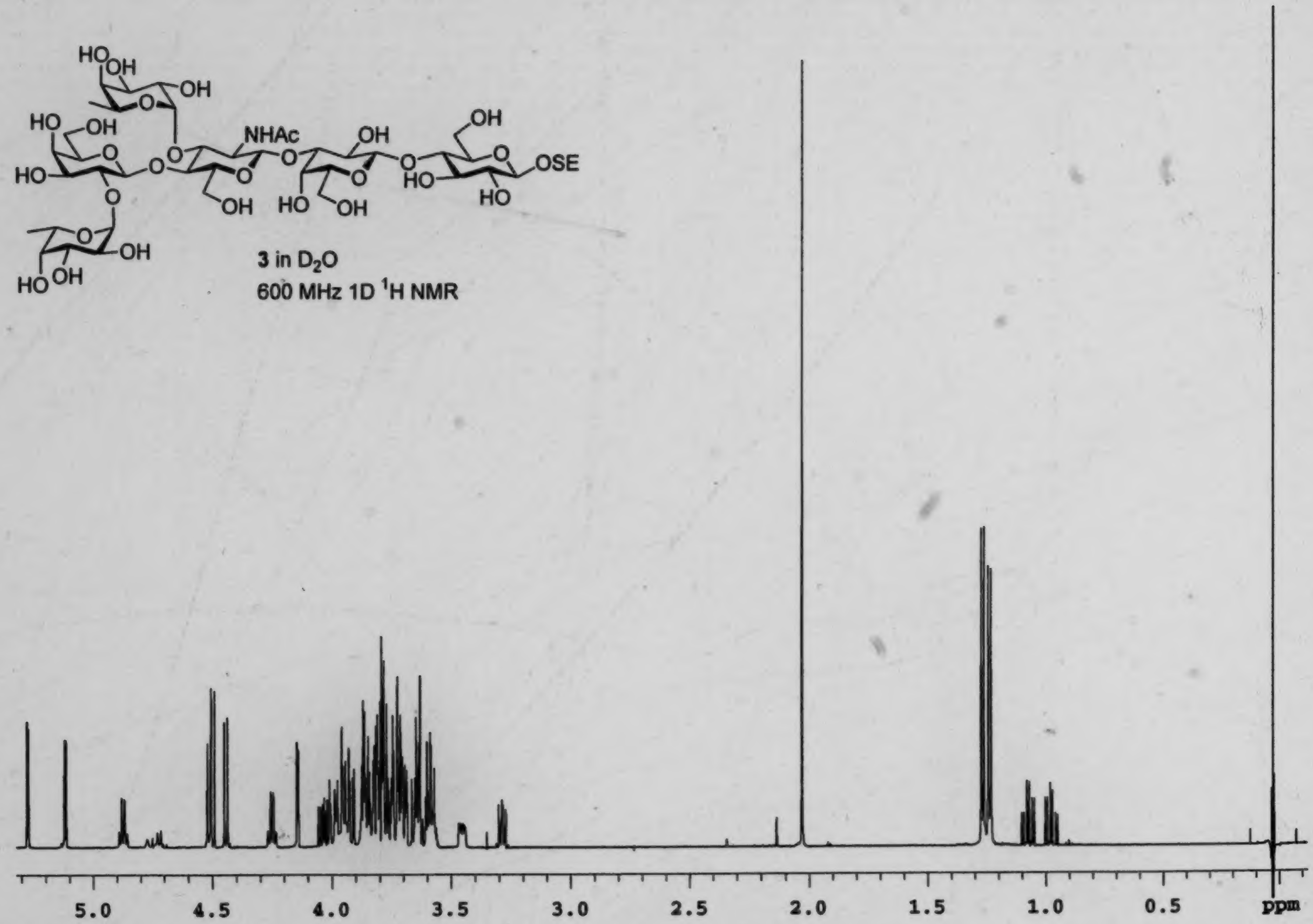
A.3. NMR Spectra of Selected Compounds



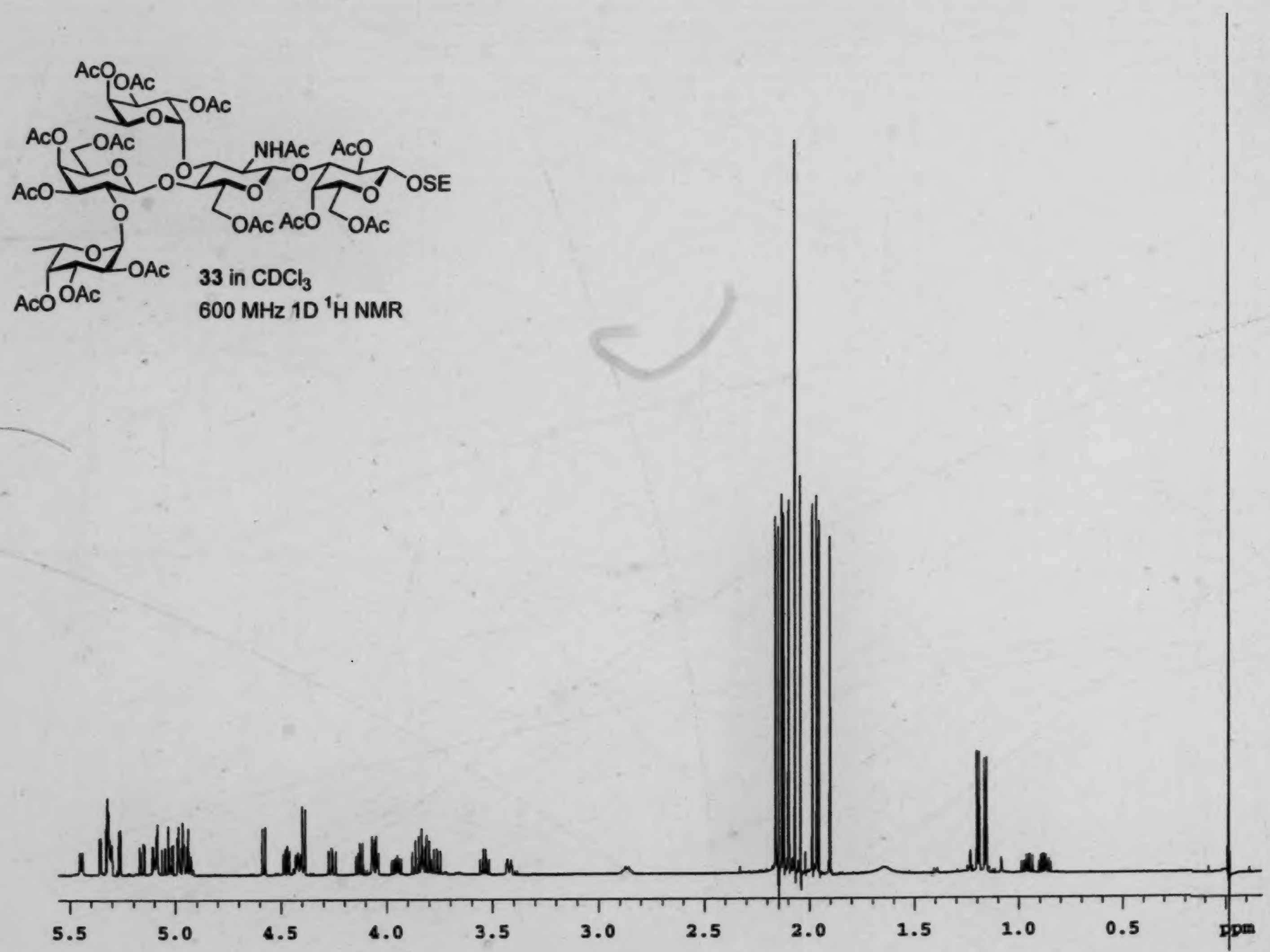
1 in D_2O
600 MHz 1D ^1H NMR



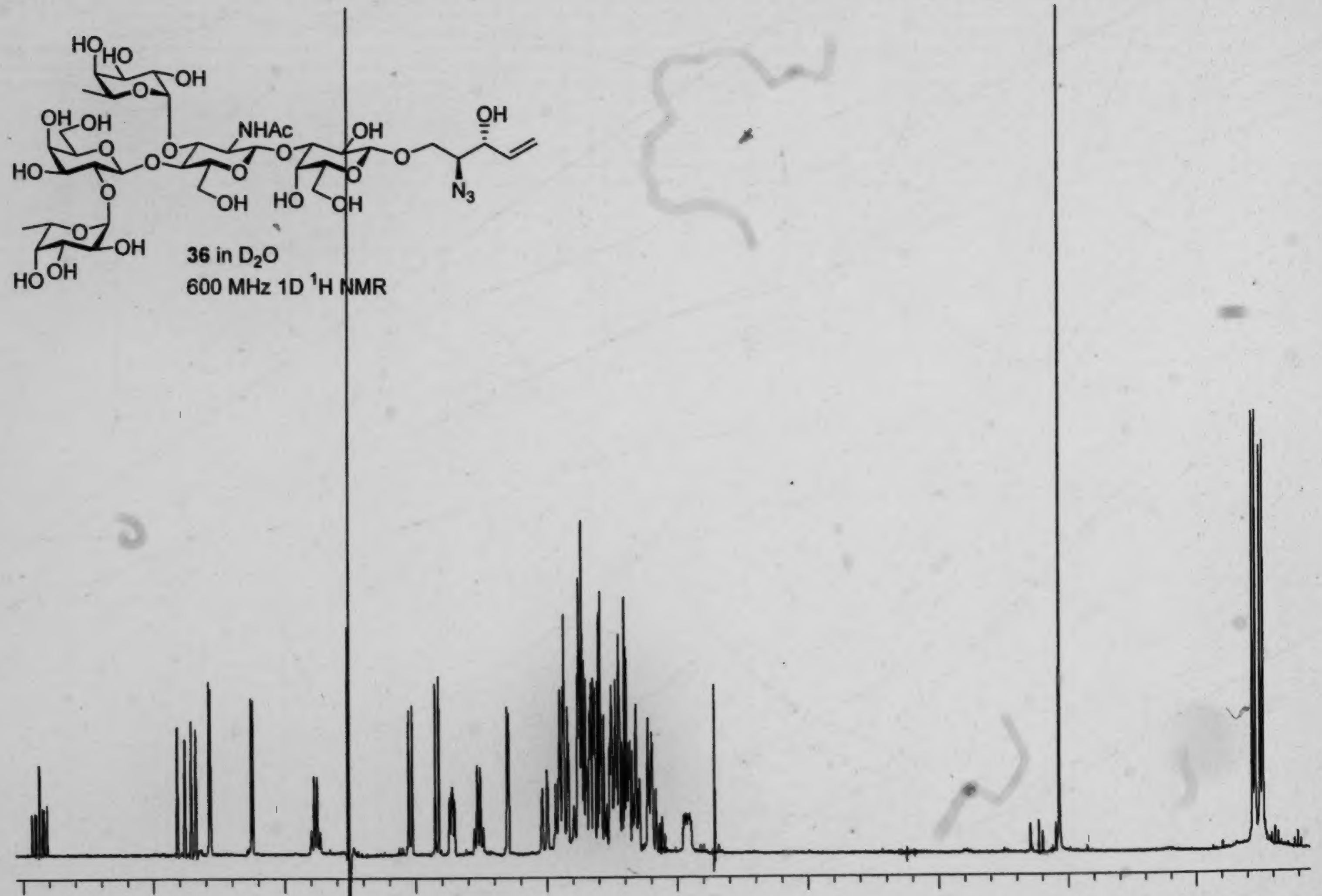
2 in D$_2$O
500 MHz 1D ^{1}H NMR



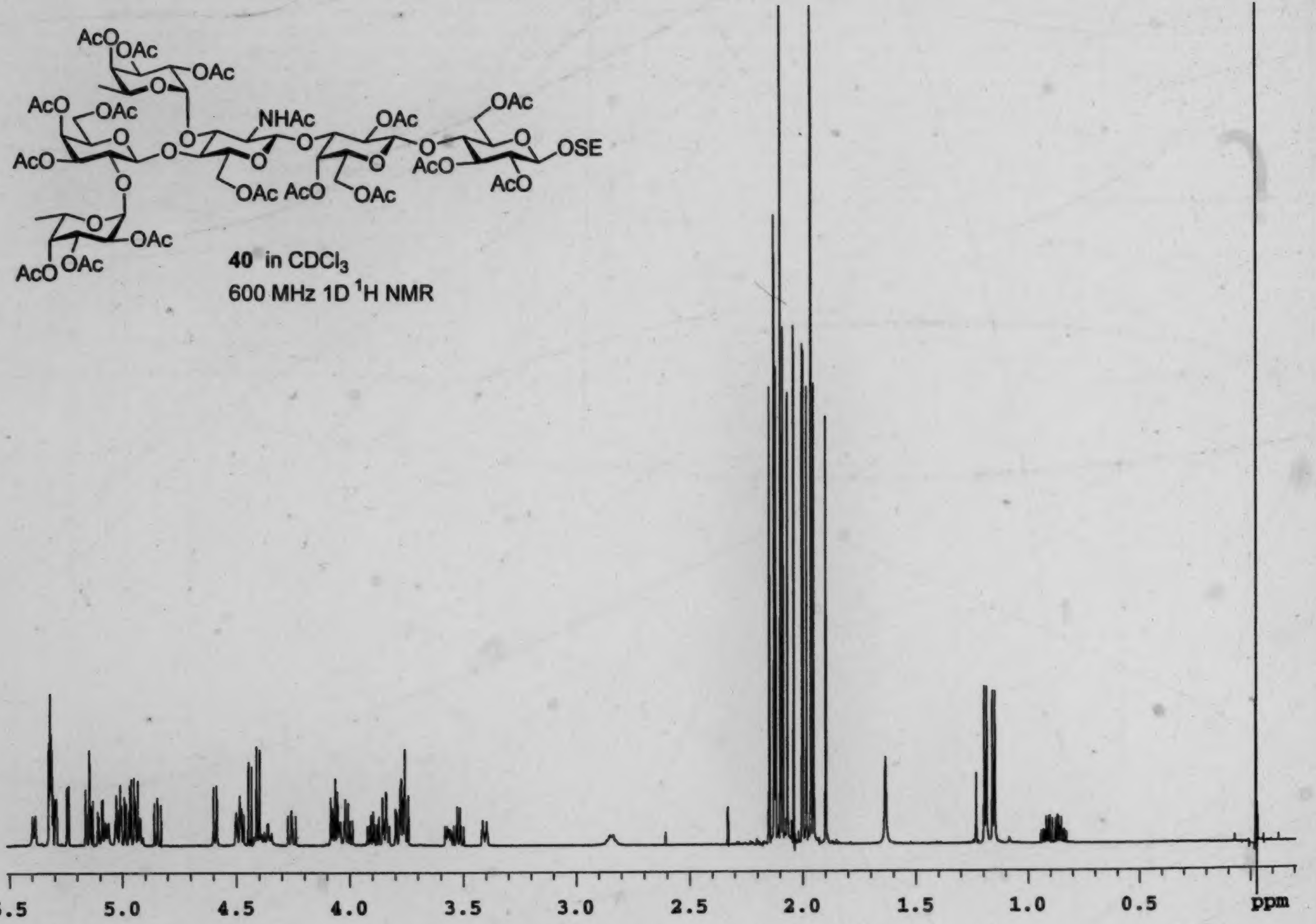
3 in D_2O
600 MHz 1D ^1H NMR



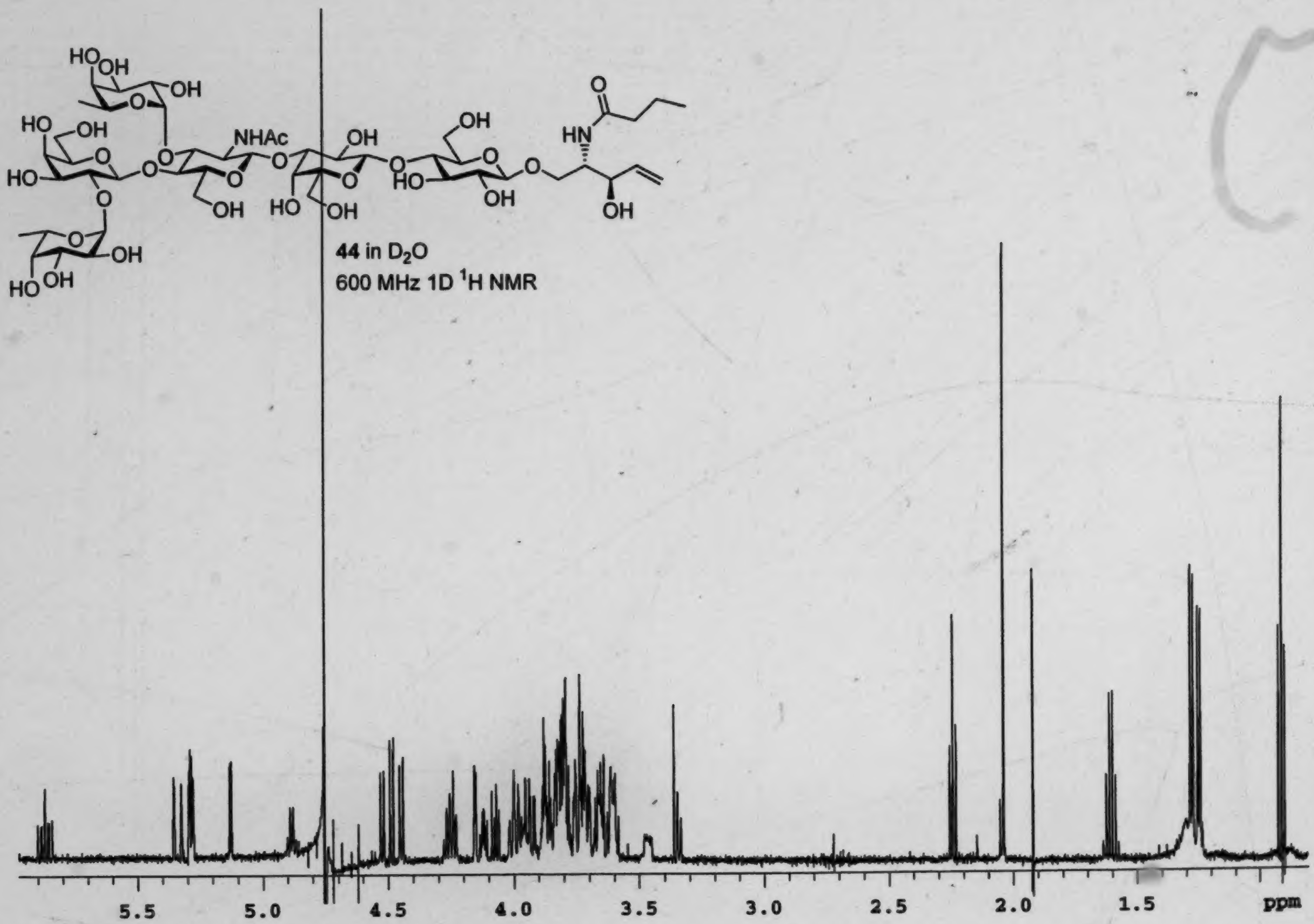
33 in $CDCl_3$
600 MHz 1D ^1H NMR



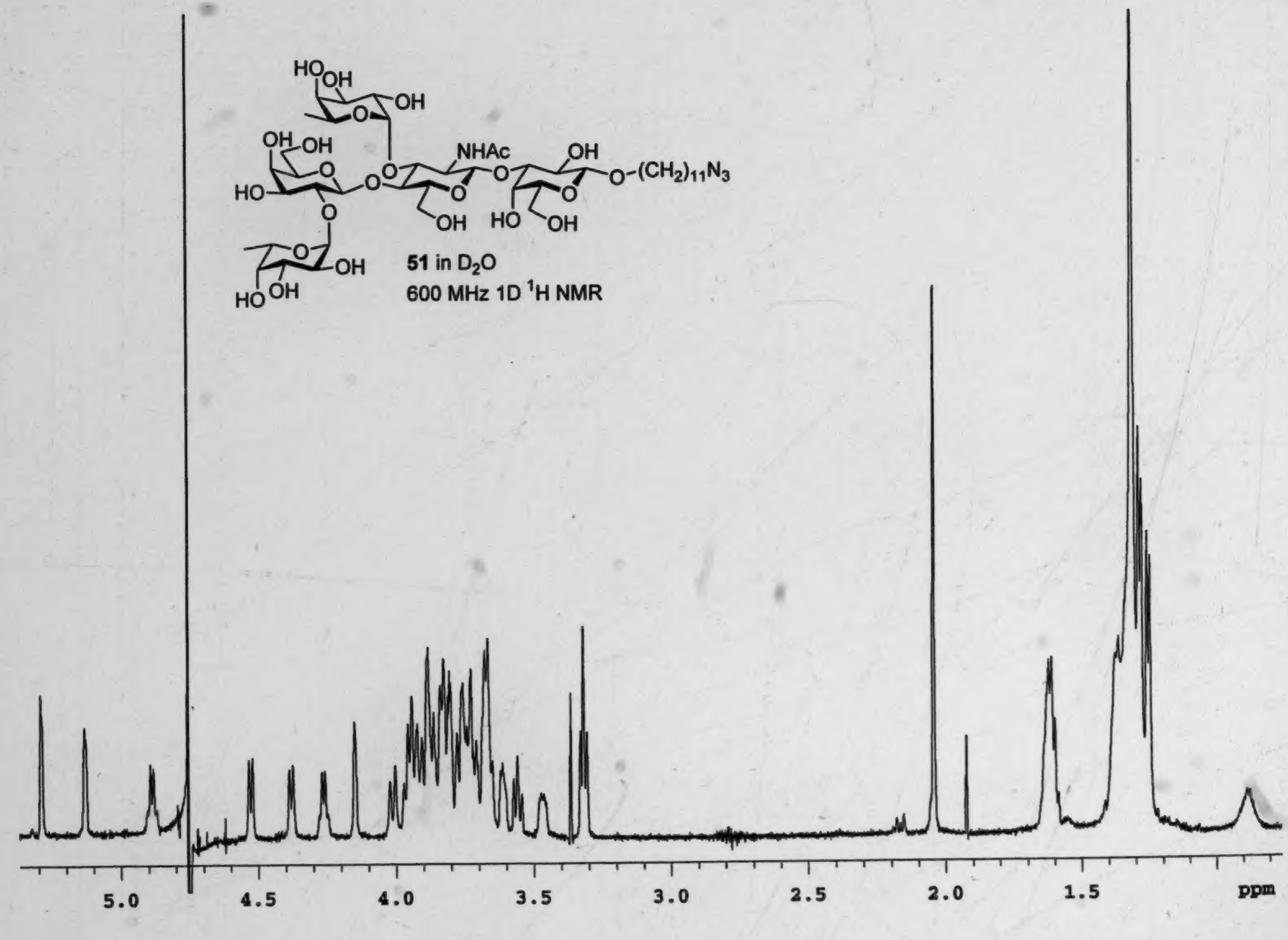
36 in D_2O
600 MHz 1D ^1H NMR



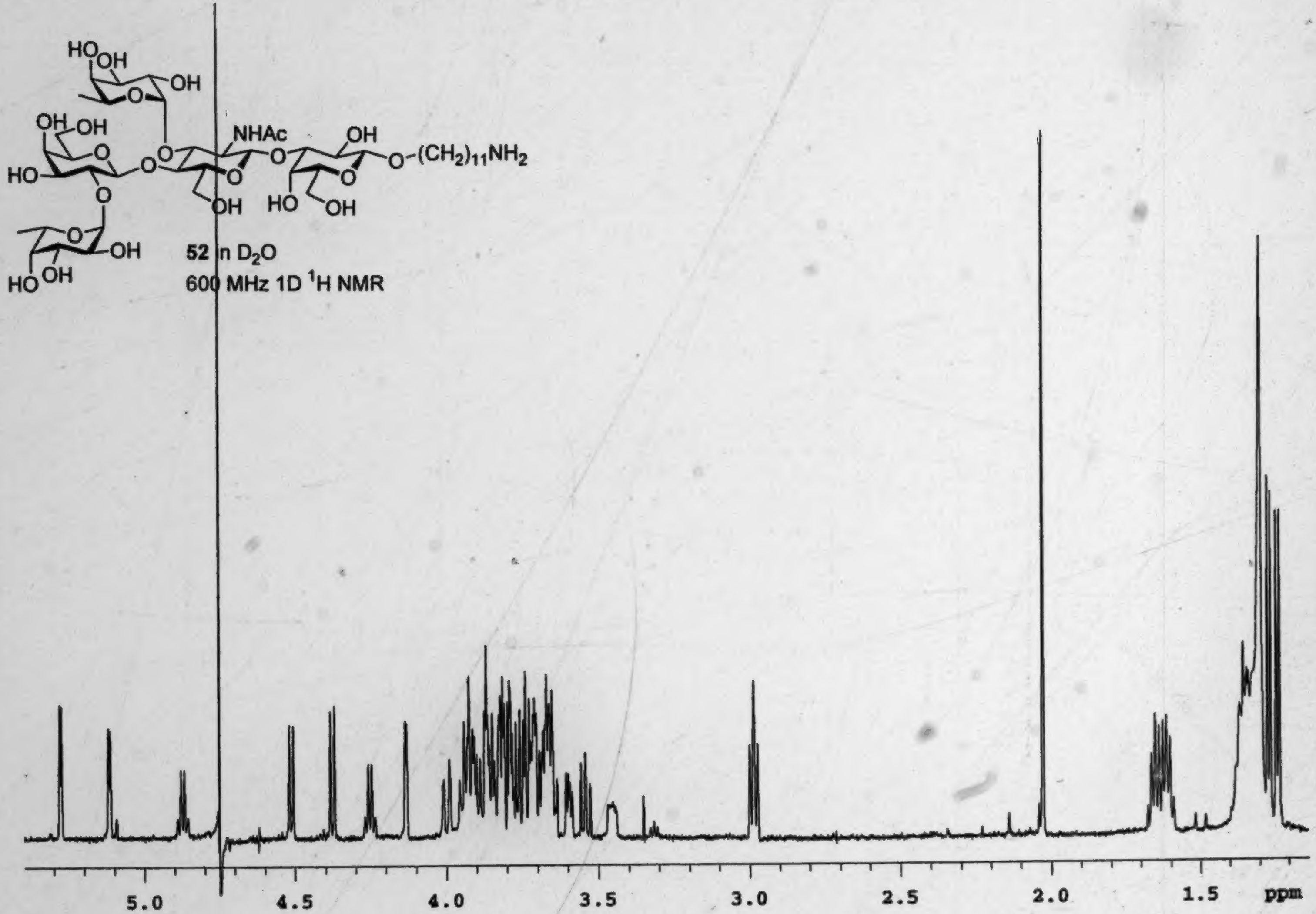
40 in $CDCl_3$
600 MHz 1D ^1H NMR



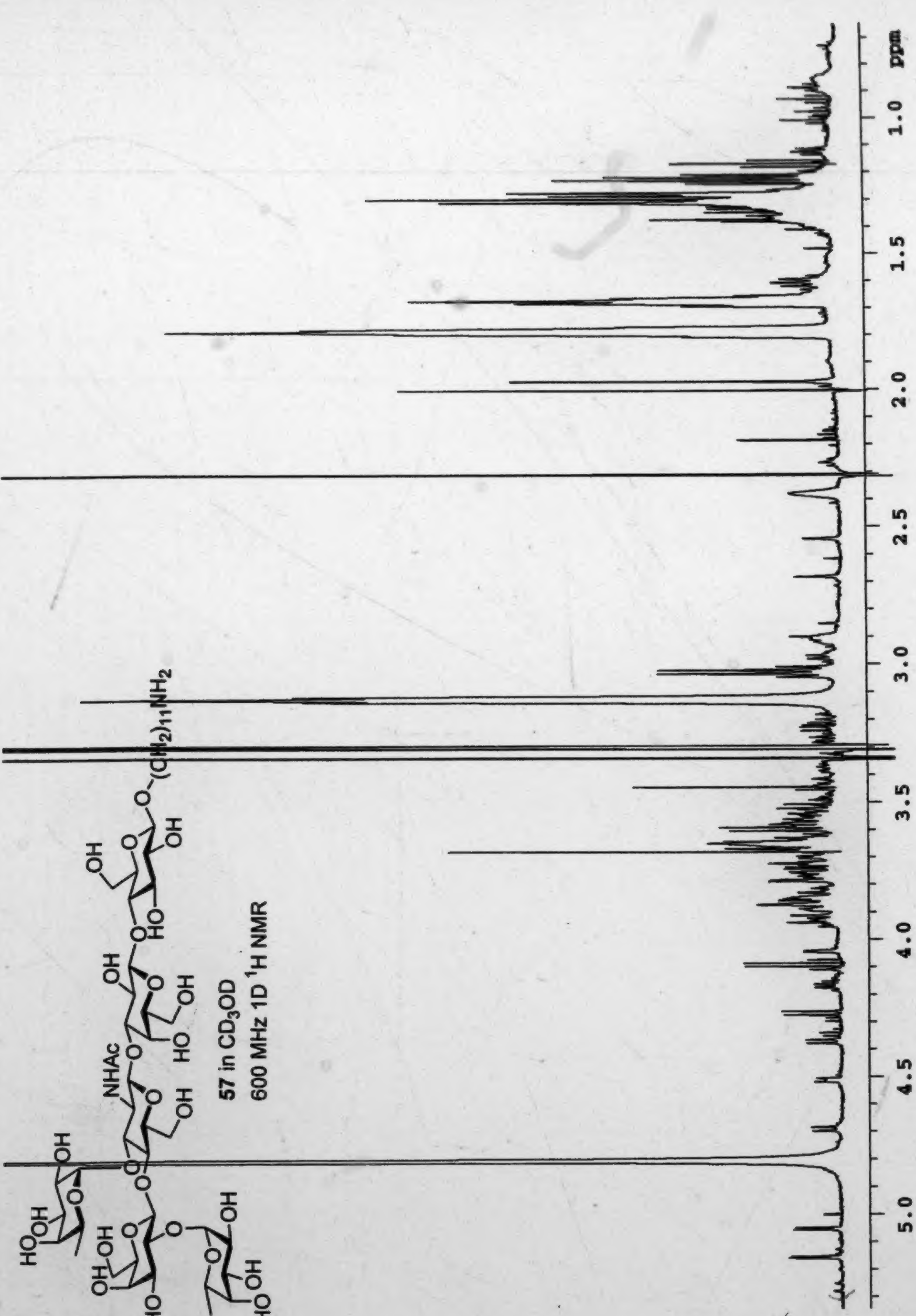
44 in D_2O
600 MHz 1D ^1H NMR



51 in D_2O
600 MHz 1D ^1H NMR



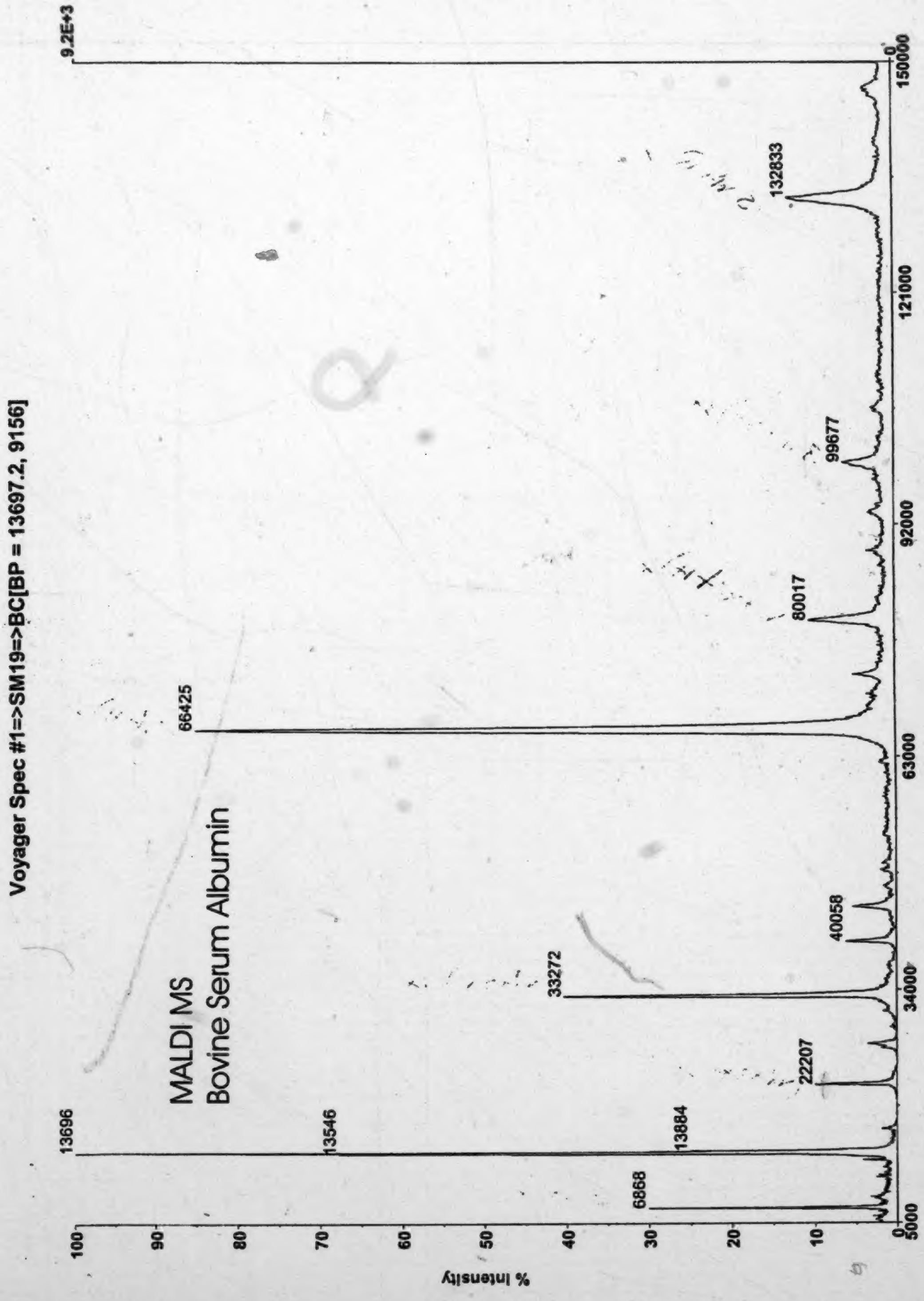
52 in D_2O
600 MHz 1D ^1H NMR

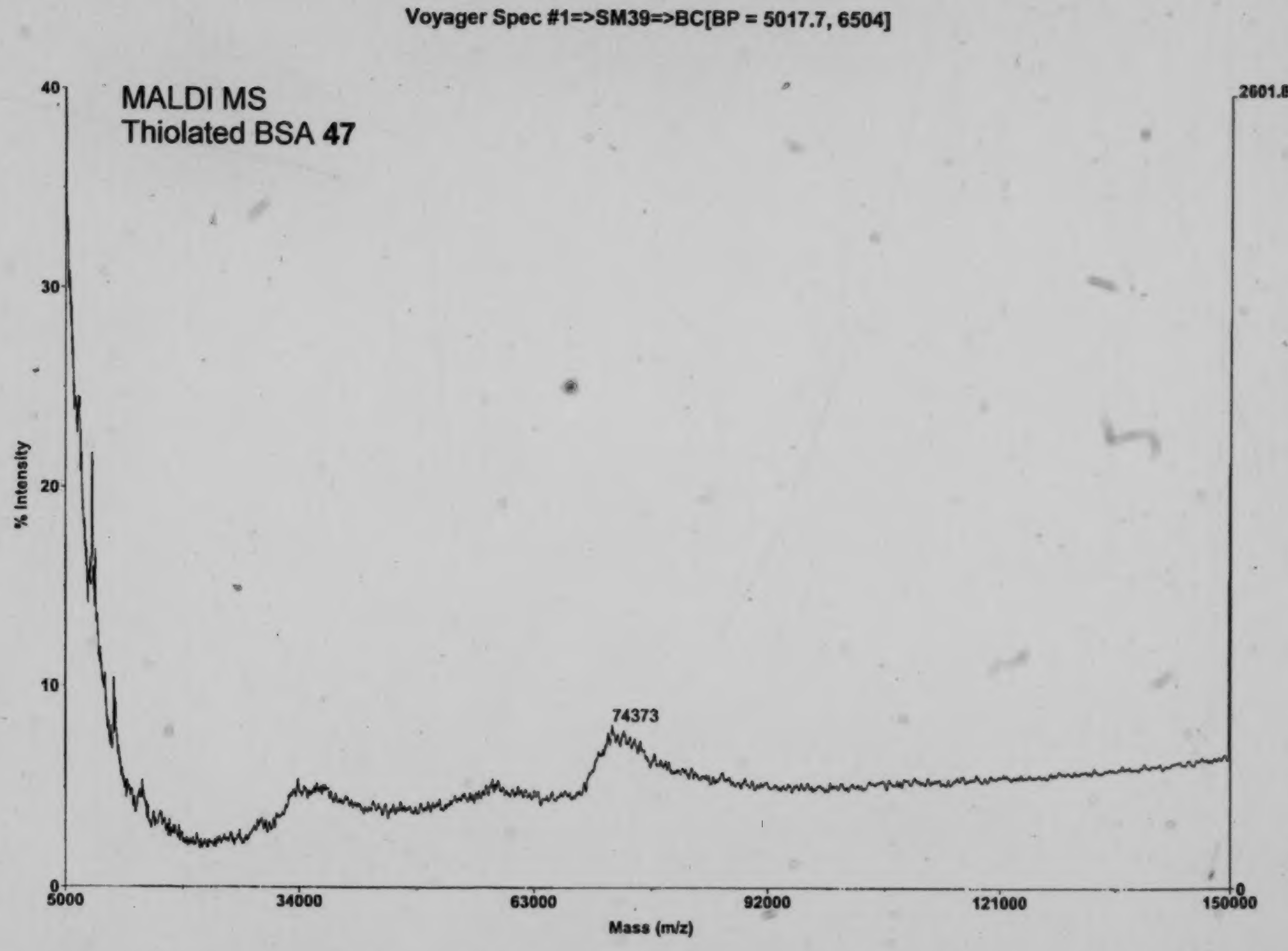


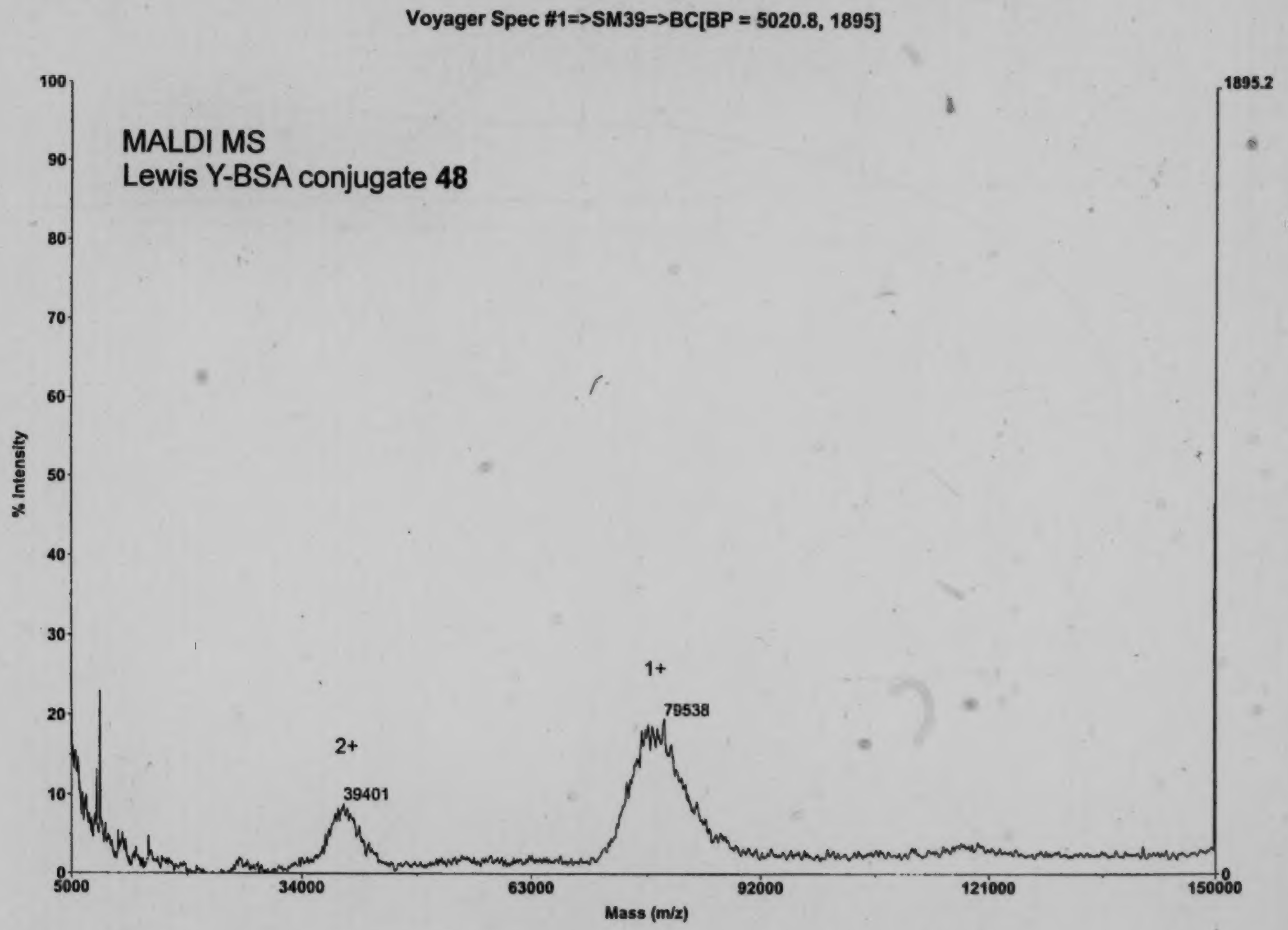
57 in CD$_3$OD

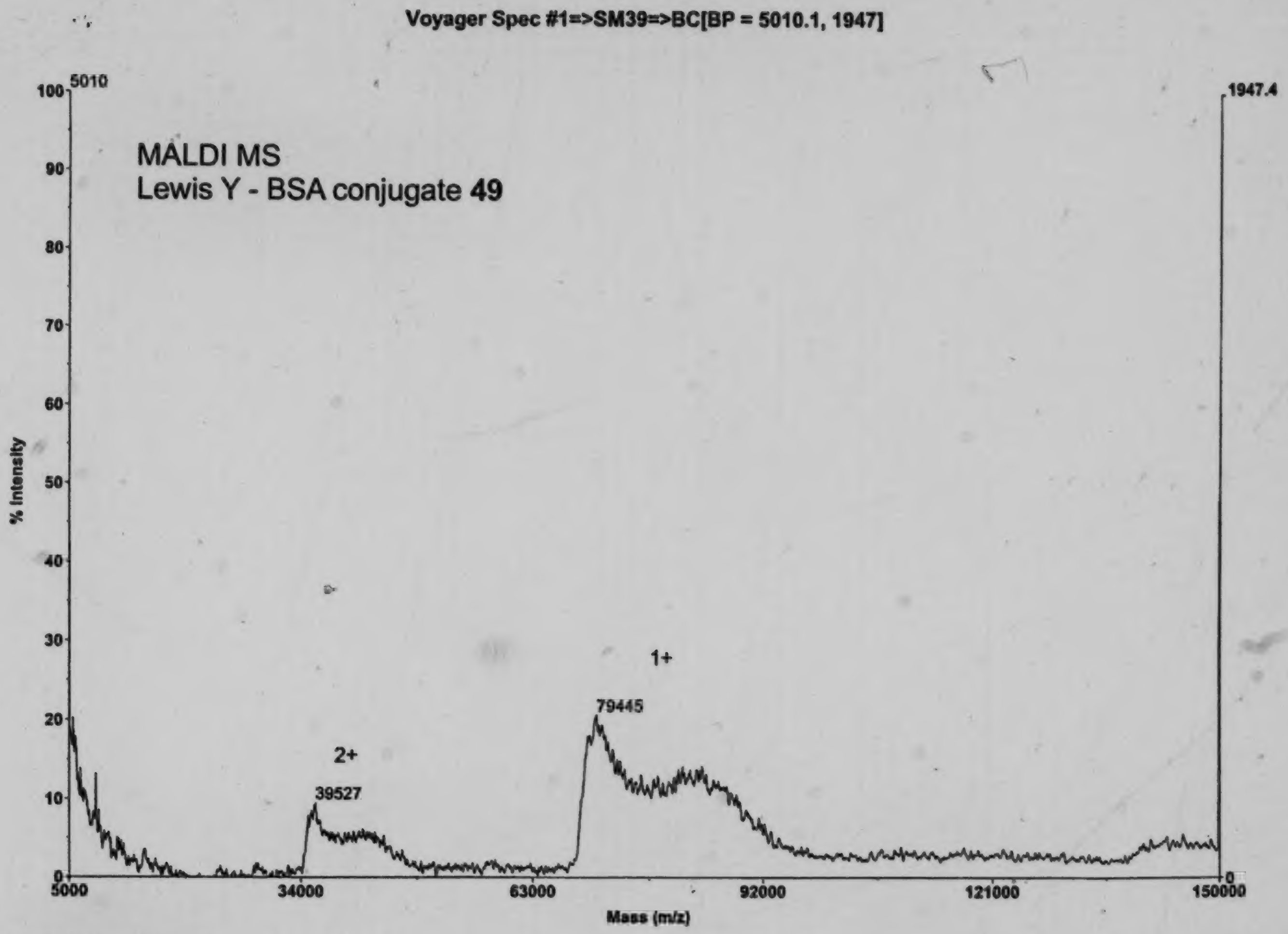
600 MHz 1D ^{1}H NMR

A.4. MALDI MS Spectra of Lewis Y – Protein Conjugates

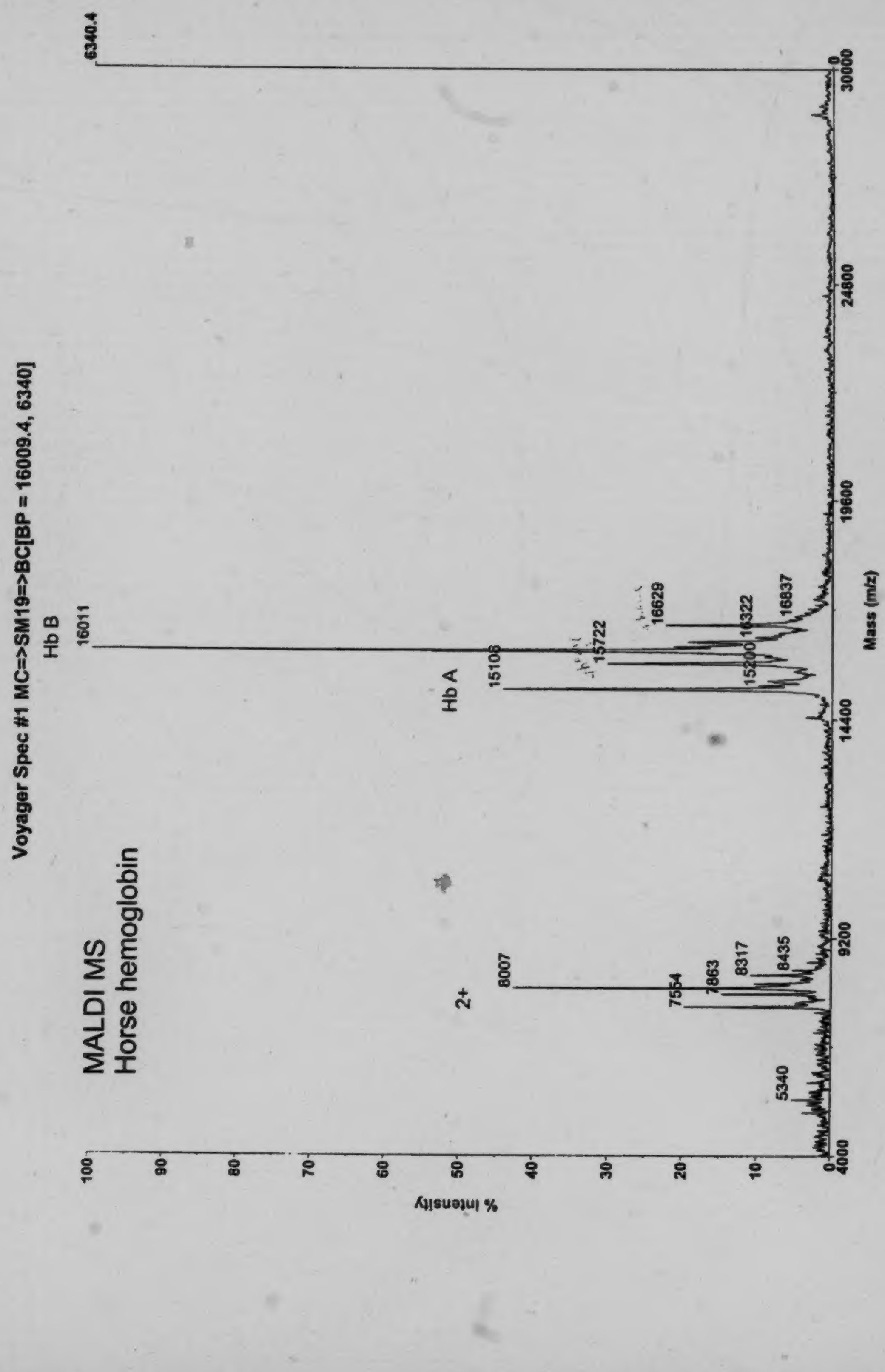


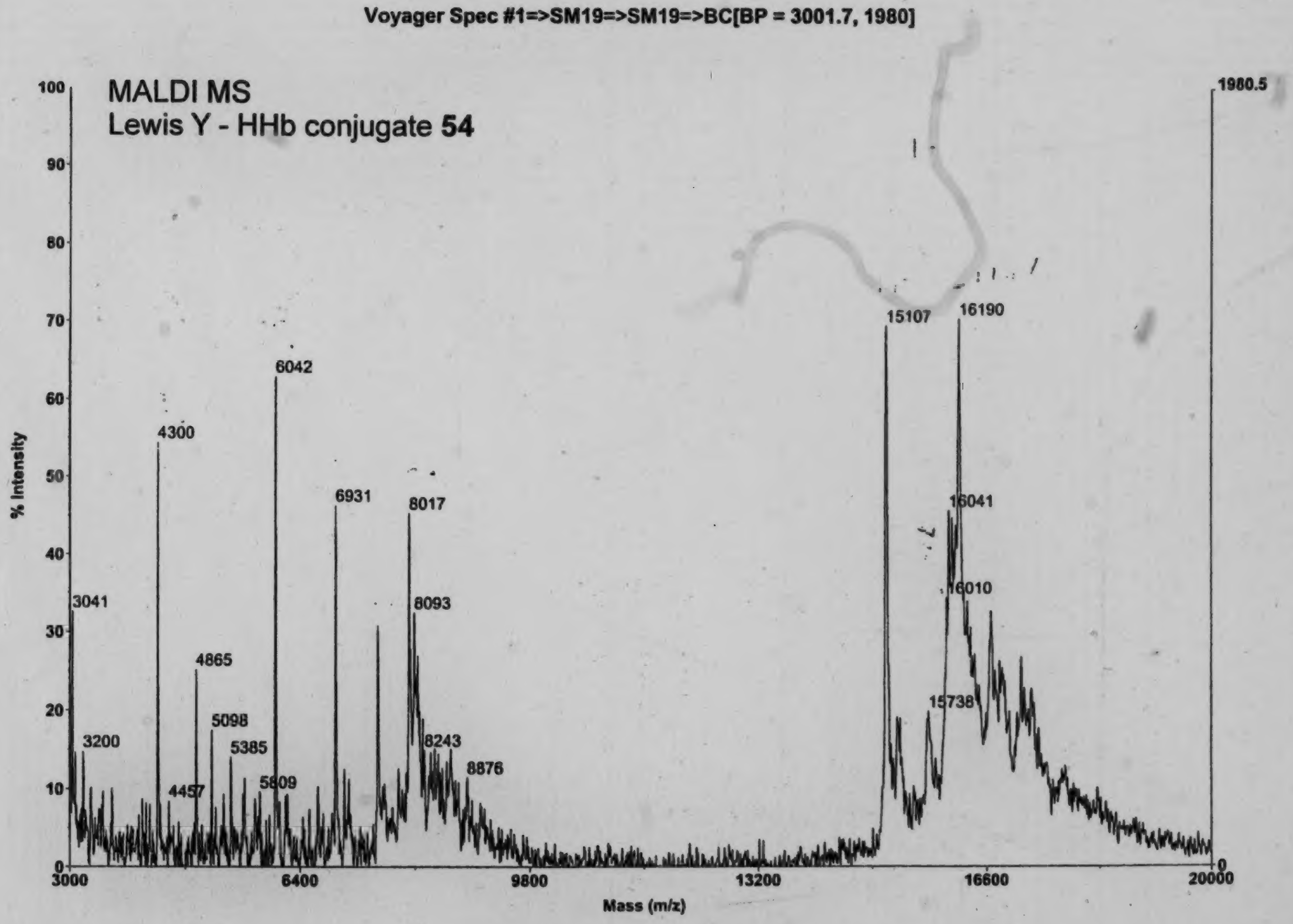

Voyager Spec #1=>SM39=>BC[BP = 5017.7, 6504]
MALDI MS
Thiolated BSA **47**
% Intensity
40
30
20
10
0
2601.8
0
74373
5000
34000
63000
92000
121000
150000
Mass (m/z)

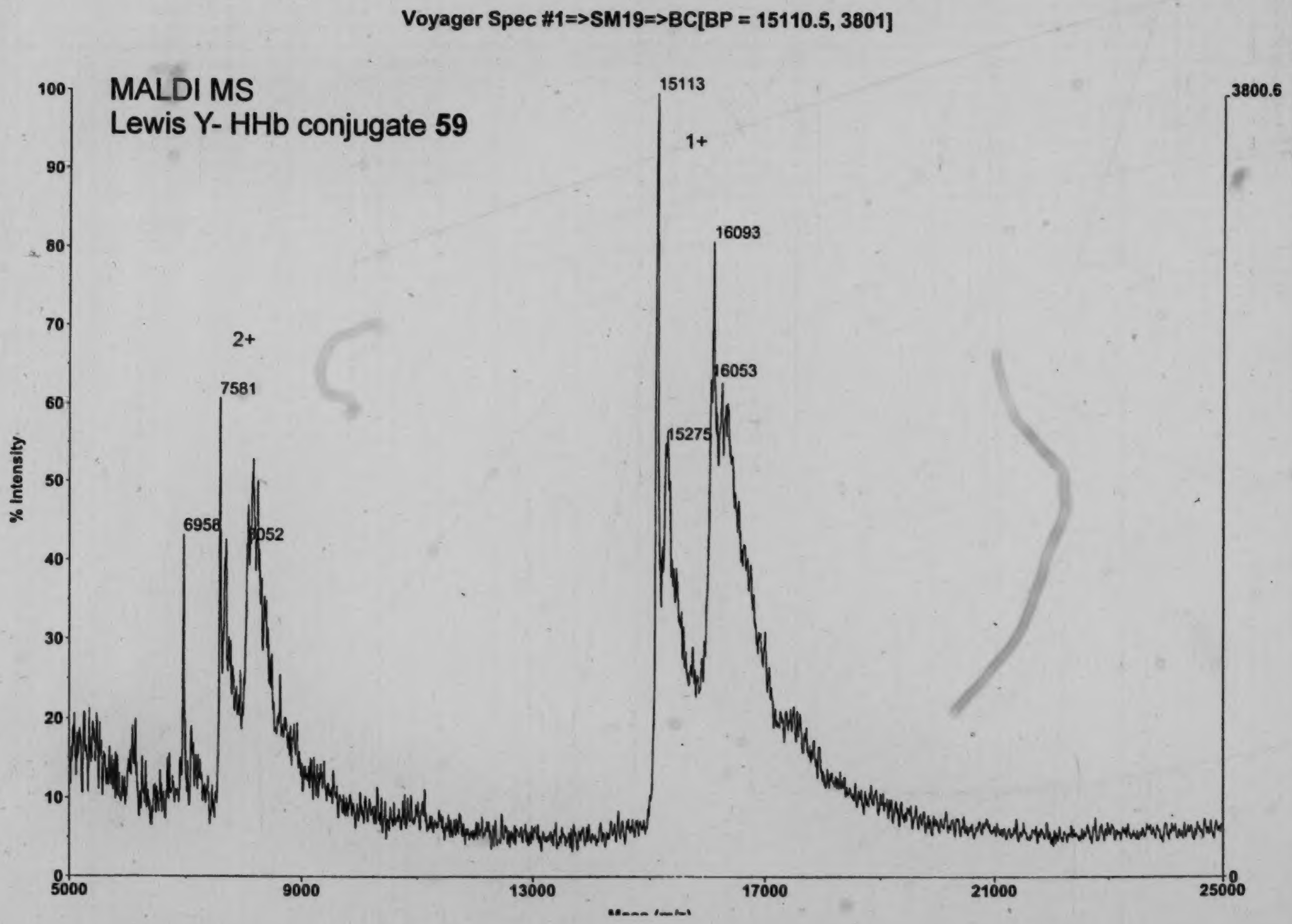

Voyager Spec #1=>SM39=>BC[BP = 5020.8, 1895]
MALDI MS
Lewis Y-BSA conjugate **48**
% Intensity
100
90
80
70
60
50
40
30
20
10
0
1895.2
0
2+
39401
1+
79538
5000
34000
63000
92000
121000
150000
Mass (m/z)



Lesley Liu LL5-79 with SA as matrix
C:\...\02090403.dat
Acquired: 09:02:00, September 04, 2002


Voyager Spec #1 MC=>SM19=>BC[BP = 16009.4, 6340]
MALDI MS
Horse hemoglobin
% Intensity
100
90
80
70
60
50
40
30
20
10
0
6340.4
Mass (m/z)
4000
9200
14400
19600
24800
30000
2+
5340
7554
8007
7863
8317
8435
Hb A
15108
15200
15722
Hb B
16011
16322
16629
16837


Voyager Spec #1=>SM19=>SM19=>BC[BP = 3001.7, 1980]
MALDI MS
Lewis Y - HHb conjugate 54
% Intensity
100
90
80
70
60
50
40
30
20
10
0
3041
3200
4300
4457
4865
5098
5385
5809
6042
6931
8017
8093
8243
8876
15107
16190
16041
16010
15738
1980.5
0
3000
6400
9800
13200
16600
20000
Mass (m/z)


Voyager Spec #1=>SM19=>BC[BP = 15110.5, 3801]
MALDI MS
Lewis Y- HHb conjugate 59
% Intensity
100
90
80
70
60
50
40
30
20
10
0
5000
9000
13000
17000
21000
25000
3800.6
0
2+
7581
6958
8052
1+
15113
16093
16053
15275